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CORRECTION

On page 702, line 3, Table I, and in the equation, line 16, Vol. 185, No. 2, August 1950, read $A - (B + C)$ for $A + (B - C)$.

ON THE MECHANISM OF THE REACTION OF NINHYDRIN WITH α -AMINO ACIDS

I. ABSORPTION SPECTRA OF NINHYDRIN AND CERTAIN DERIVATIVES*

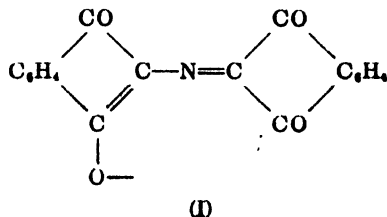
By DOUGLAS A. MacFADYEN

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(Received for publication, January 21, 1950)

The present absorption spectra pertain to the mechanism of the reactions of ninhydrin with α -amino acids. They represent ninhydrin, triketohydrindene, the quinoxaline (ketophenazine) derivative, ninhydrin, ureide, ninhydrin guanide, the compound formed by condensing ninhydrin with dimethyldihydroresorcinol, the substance responsible for the purple color which appears when proteins and their components and primary amines generally react with ninhydrin, and diketohydrindamine-diketohydrindylidene.

The purple dye, according to Ruhemann (1) and Harding and MacLean (2), was the ammonium salt of diketohydrindamine-diketohydrindylidene. MacFadyen (3) and Moore and Stein (4) have shown that the sodium salt solution yields the same purple color. I have found that aqueous solutions of the sodium salt and of the potassium salt have one set of molar absorption coefficients. Therefore, the purple color is independent of the nature of the cation and is to be attributed to the anion of diketohydrindamine-diketohydrindylidene, which I propose to call *Ruhemann's purple*. One of at least four of its resonance forms is shown in formula I.



Apparatus—

1. Spectrophotometer, model DU of the National Technical Laboratories. The wave-length scale at $\lambda = 546.1, 404.7$, and $253.7 \text{ m}\mu$ checked, by means of a mercury arc lamp, within $0.3 \text{ m}\mu$.

* Supported by the Otho S. A. Sprague Memorial Institute.

† Some of the work was carried out at the Alfred I. du Pont Institute in Wilmington, Delaware, and at the Army Medical School in Washington.

2. Cuvettes, silica, for the ultraviolet, and Corex for the visible region of the spectrum. The length of the light path was 1 cm.

Preparation of Ninhydrin and Derivatives

The purity of substances previously reported was checked by elementary analysis and melting points.

Ninhydrin was obtained from the Eastman Kodak Company, purified by recrystallization from 0.1 N sulfuric acid, extraction with ethyl acetate, and recrystallization from water, according to Ruhemann ((5) p. 1446).

The quinoxaline derivative was prepared according to Ruhemann (5, 6).

Ninhydrin ureide was prepared according to Polonovsky, Gonnard, and Glotz (7) as modified by Van Slyke and Hamilton (8).

Ninhydrin guanide was prepared according to Ruhemann ((6) p. 2026), under conditions specified by Van Slyke and Hamilton (8) for the ureide.

Compound of Ninhydrin with Dimethyldihydroresorcinol—A solution of ninhydrin, 1.82 gm. (0.01 M) and dimethyldihydroresorcinol, 3.0 gm. (0.022 M), in 200 ml. of 0.1 N sulfuric acid was heated to boiling for 15 minutes. On cooling, a colorless, or faintly peach-colored, precipitate was obtained. It was recrystallized from dilute sulfuric acid, washed with water at 5°, and yielded 1.37 gm. after drying in air to constant weight. There was no loss in weight on heating at 105° for 1 hour. The crystals melted sharply, with decomposition, at 210–212° on a stage melting point apparatus during an elevation of temperature of 1° every 8 seconds. They turned yellow and decomposed if kept at 180°. Calculated for $C_{17}H_{16}O_8$ (300.3), C 67.96, H 5.33 per cent; observed, C 67.80, H 5.11.

Sodium Salt of Diketohydrindamine-Diketohydrindylidene— α -Alanine, 0.65 gm., and ninhydrin, 0.3 gm., were dissolved in 100 ml. of 0.1 M McIlvaine's buffer mixture at pH 5.0, brought to boiling for 3 minutes, and allowed to cool for 1 hour. The precipitate was dried in air, extracted with benzene until the extract showed no red color, recrystallized from hot water, and dried at 110° to constant weight. Elementary analysis gave results previously indicated (3, 4). The melting point was above 310°. The substance is insoluble in benzene, ether, chloroform, and very slightly soluble, about 10 mg. per liter, in water. The yield at pH 5.0 is the maximum, approximately 50 per cent, assuming that 2 moles of ninhydrin are required per mole of amino acid.

Potassium Salt of Diketohydrindamine-Diketohydrindylidene—This was prepared as for the sodium salt, in 0.1 M McIlvaine's buffer solution; potassium was substituted for sodium. Calculated for $C_{18}H_{16}O_7NK$ (341.3) C 63.33, H 2.35, N 4.13, K 11.46 per cent; observed, C 63.02, H 2.42, N 4.15, K 11.78. The melting point is above 310°.

Diketohydrindamine-Diketohydrindylidene · 2H₂O—A suspension of the so-

dium salt in 0.001 N sulfuric acid was extracted with benzene. The material in benzene was dried immediately on a steam bath. The red-colored solid decomposed at 110–120°. Calculated for $C_{18}H_{10}O_4N \cdot 2H_2O$ (339.3), C 63.71, H 3.86, N 4.13 per cent; observed, C 64.44, H 3.67, N 4.14.

Method of Presentation of Absorption Data

The absorption spectra are plots of the molar absorption coefficient, ϵ , when multiplied by 10^{-2} , against the frequency, $\bar{\nu}$, in wave number per

TABLE I
Some Parameters of Absorption Spectra of Ninhydrin and Some Monoindane Derivatives at 25° in Various Solvents

Solvent		$\epsilon_{max} \times 10^{-2}$	λ_{max}	Reference		$\epsilon_{max} \times 10^{-2}$	λ_{max}	Reference
			m μ				m μ	
Water	Ninhydrin dimethyldihydroresorcinol derivative	37.2	232	Fig. 1	Ureide	0.2	341	Fig. 4
		18.0	286	" 5		1.7	291	" 4
		36.0	227	" 5		11.7	253	" 4
		23.6	248	" 5		10.0	253	" 4*
Ethanol	Ninhydrin		357	(10)	Ninhydrin	0.1	357	Fig. 1
						44.0	228	" 1
95% ethanol	"	0.1	362	(7)	Ureide	0.14	346	(7)
		10.4	247	(7)		1.6	292	(7)
		36.3	226	(7)		11.8	248	(7)
		36.0	234	Fig. 2		0.1	549	Fig. 2
Concentrated H_2SO_4	Ureide	4.0	365	" 4	Triketohydrindene	3.0	350	" 2
		30.2	239	" 4		26.4	277	" 2

* Otherwise the same as the ureide.

cm., when multiplied by 10^{-2} . $\bar{\nu}$ was computed¹ from the wave-length, λ , in m μ , by the expression, $\bar{\nu} = 10^7/\lambda$. ϵ was computed from the equation for Beer's law. The criterion for applicability of Beer's law was the absence of any deviation in excess of 1 per cent of the calculated value for at least three concentrations in an over-all 10-fold range. Beer's law was assumed in the case of Ruhemann's purple and diketohydrindamine-diketohydrindylidene in organic solvents. In them, decolorization is too rapid for unambiguous measurements of optical density, but not for estimating the order of magnitude of the absorption coefficients.

¹ Reasons for choice of this method of presentation are given by West (9).

Discussion of Absorption Spectra

Ninhydrin (1,2,3-Indantrione Hydrate) (Fig. 1; Table I)—The complete absorption curve of ninhydrin at pH 4.8 shows several plateaus, some of which may represent absorption maxima. The main maximum is the one at $\bar{\nu} = 43,100$; $\lambda = 232$ m μ . These and previous data agree (7, 10).

The absorption curve does not change with pH if recorded immediately after dissolving the ninhydrin. On standing at room temperature, the maximum at 232 m μ disappears, as is shown for pH 9. The change is the more rapid the higher the pH, and is probably caused by internal oxidation-reductions (5), shown in II.

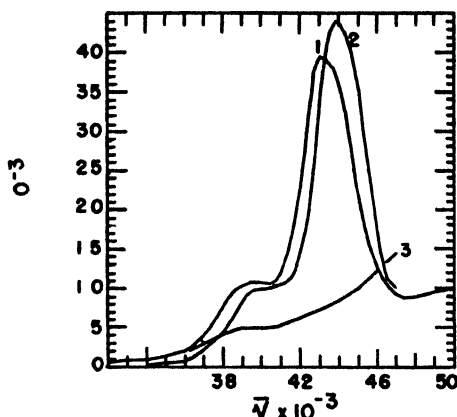
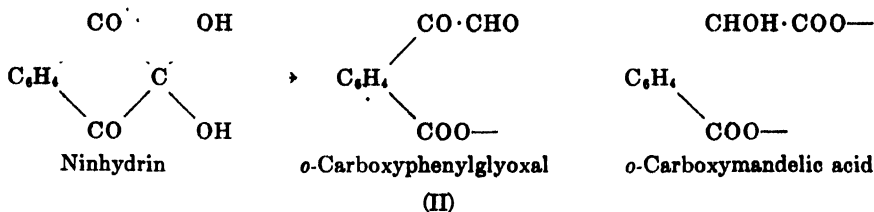


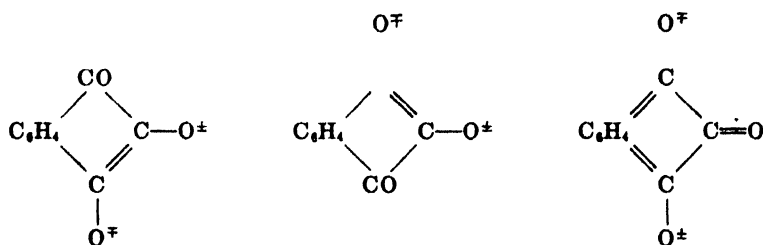
FIG. 1. Absorption curves for ninhydrin. Curve 1, in water, at pH 4.8; Curve 2, in ethanol; Curve 3, at pH 9.0 after 24 hours at 25°.

Triketohydrindene (1,2,3-Indantrione) (Fig. 2; Table I)—Its red color appears when ninhydrin is heated to 120° (5) or when ninhydrin is dis-



solved in concentrated sulfuric acid. The absorption curves vary reversibly with the water content of the sulfuric acid, representing triketohydrindene at specific gravity 1.80, and ninhydrin at specific gravity 1.52. By analogy with the resonance forms suggested by Ferguson (11) for triketopentane, the red color is assigned the structure shown in III.

Quinoxaline Derivative (Indanone-2,3-quinoxaline) (Fig. 3; Table I)—The canary-yellow color is common to most phenazines. On standing



(III) Triketohydrindene

in ethanol solution at room temperature without exposure to air or in aqueous suspension with sodium hydrosulfite, the quinoxaline changes

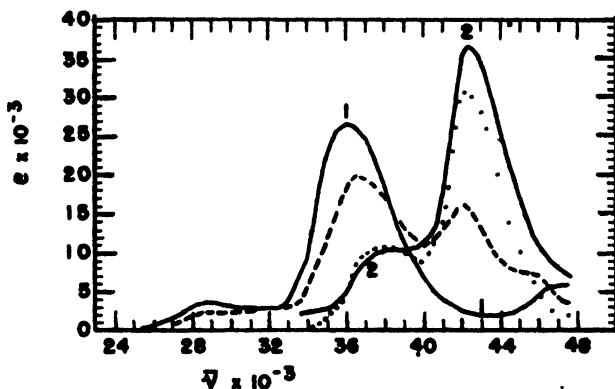


FIG. 2. Conversion of ninhydrin to triketohydrindene in sulfuric acid. Curve 1, triketohydrindene, sp. gr. 1.80; dash line, sp. gr. 1.73; dotted line, sp. gr. 1.64; Curve 2, ninhydrin, sp. gr. 1.52.

color from yellow to blue. The blue color vanishes on shaking air into the solution.

Ninhydrin Ureide and Guanide (Fig. 4; Table I)—Since all the evidence (5, 6) is for the view that only the 2-carbonyl group of ninhydrin is hydrated, condensation products will form by linkage to that group. Therefore, the simplest form of the ureide (see Polonovsky *et al.* (7)) is that shown in IV. The formula for the guanide comes from Ruhemann (6). Both substances are inert to *o*-phenylenediamine in dilute acid solution.

Formula IV is assigned to the ureide in sulfuric acid, specific gravity 1.80. The contour of the absorption curve in the ultraviolet (which changes slowly as the yellow color of the solution fades) is very similar to that of ninhydrin in sulfuric acid, specific gravity 1.52, and the param-

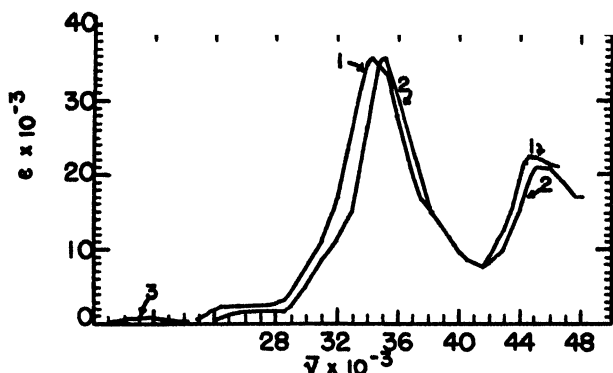


FIG. 3. Absorption curve for the quinoxaline derivative of ninhydrin. Curve 1, in 95 per cent ethanol; Curve 2, in cyclohexane; Curve 3, in 95 per cent ethanol; after 18 hours anaerobically at 25°, a new fundamental band appears in Curve 1.

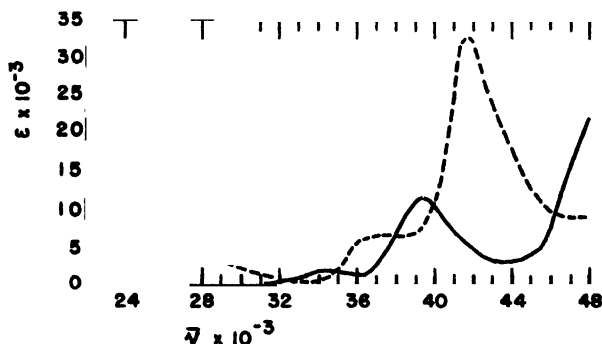


FIG. 4. Absorption curves for ninhydrin ureide and guanide. The solid line represents ureide and guanide in water (see the text for slight differences); the dash line, ureide in concentrated H_2SO_4 , sp. gr. 1.80.

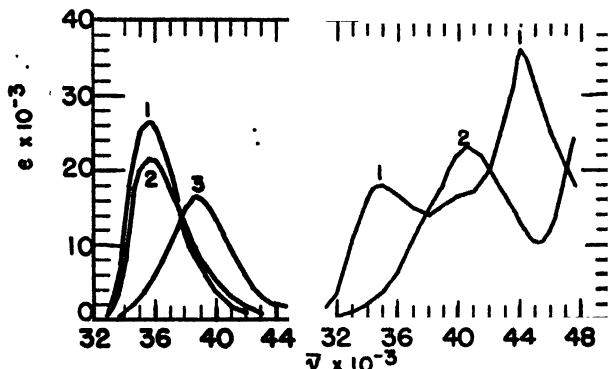
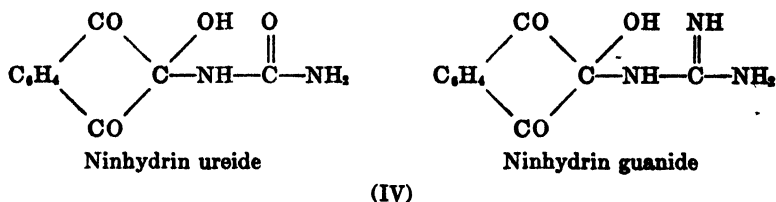


FIG. 5. Absorption curves of dimethyldihydroresorcinol (on the left) and the compound formed from it and ninhydrin (on the right), at varied pH. On the left, Curve 1, pH greater than 6.8; Curve 2, pH 5.7; Curve 3, pH less than 3.5; on the right, Curve 1, pH greater than 6.5; Curve 2, pH less than 2.2.

eters are of the same order of magnitude. Since it has been shown (8) that the ureide crystallizes with 1 mole of water, the spectrum in water



and the curve shown by Polonovsky *et al.* (7) are assigned to the hydrated ureide. The change in absorption caused by dehydration is reversible

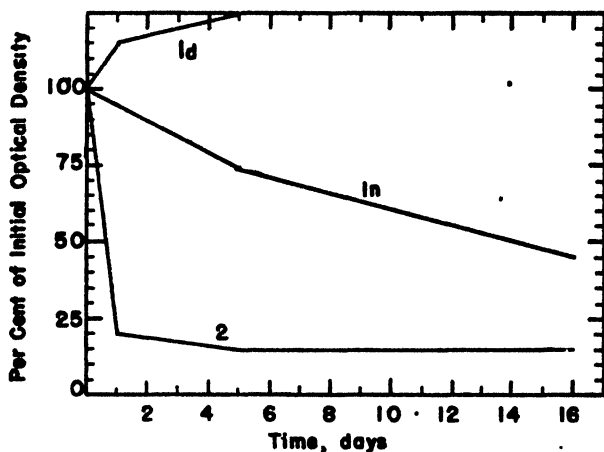
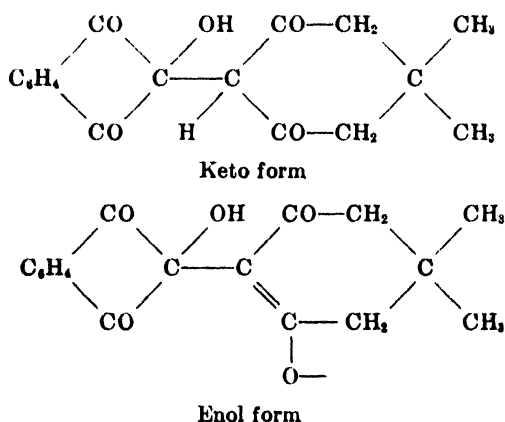


FIG. 6. Rate of hydrolysis of the compound formed from ninhydrin and dimethyldihydroresorcinol at 25°, pH 9.0. Curve 1d dimethyldihydroresorcinol component; Curve 1n, ninhydrin component; Curve 2, ninhydrin alone; measured at wavelengths of 280, 230, and 232 $m\mu$ respectively.

and is analogous to the change from triketohydrindene to ninhydrin in similar concentrations of sulfuric acid.

Compound Formed by Condensing Ninhydrin with Dimethyldihydroresorcinol (2-OH-1,3-Indandione-2,2'-(1,3-cyclohexanedione-5,5-dimethyl) (Fig. 5; Table I)—At appropriate pH, the compound reveals the absorption curves due to ninhydrin and dimethyldihydroresorcinol, with allowance for slight shifts in the maxima. In alkaline solution it is unstable, less stable than the ureide and Ruhemann's purple, decomposing into its components (ninhydrin changes to *o*-carboxyphenylglyoxal) (Fig. 6). As the pH is varied, the compound reveals both the curves for dimethyldihydroresorcinol, the keto form in more acid solution, the enol form in less acid solution, and transitional curves, at pH from 2.2 to 6.5, not shown

in Fig. 5. According to Bennett (*cf.* (12)) the enol form is ionized. The formulae shown in V are assigned to the compound. The compound is inert to *o*-phenylenediamine except upon hydrolysis, which occurs readily at most pH values when the solutions are heated.



(V) Ninhydrin dimethyldihydroresorcinol derivative

Ruhemann's Purple (Indandione-2-N-2'-indanone Enolate) (Fig. 7; Table II)—Absorption spectra obtained from solutions of the sodium salt are identical with those from reaction mixtures of ninhydrin with primary amines (3, 4). Earlier work has been extended to include ultraviolet absorption and the potassium salt. The sodium salt has been isolated from the reaction of ninhydrin with several more amino acids, α -alanine, arginine, aspartic acid, and leucine. In each case, at all wave-lengths studied, Beer's law was obeyed. These facts, together with the substantial yield of Ruhemann's purple from reaction mixtures of ninhydrin and α -alanine, lead me to conclude that Ruhemann's purple is the color formed in the reaction of ninhydrin with proteins, their components, and primary amines generally.

Formula I is assigned to Ruhemann's purple because aqueous alkaline solutions of the ammonium, sodium, and potassium salts all have the same purple color. In contrast to ninhydrin, the ultraviolet absorption is constant at pH 10, 25°, for weeks, except in dilute NH₄OH, in which the purple color fades rapidly as silky yellow crystals precipitate. A report on the yellow substance will be given later. At pH 1, the purple solution decolorizes and yields the absorption curve of ninhydrin, the optical density at 232 m μ corresponding to the formation of 2 moles of ninhydrin for each mole of Ruhemann's purple. Tests with *o*-phenylenediamine are inconclusive because Ruhemann's purple hydrolyzes in acid solution, the

faster the lower the pH, particularly on heating. The quinoxaline derivative of ninhydrin is deposited in such tests.

The purple color in ethanol, acetone, or pyridine solutions fades gradually, in hours at room temperature.

Hydrosulfite decolorizes Ruhemann's purple; the effect of an excess is irreversible at the pH tested, 4 to 8; oxygenation restores the color but not to its original intensity.

The absorption spectrum obtained when the sodium salt is dissolved in concentrated sulfuric acid, sp. gr. 1.80, I attribute to the formation of an

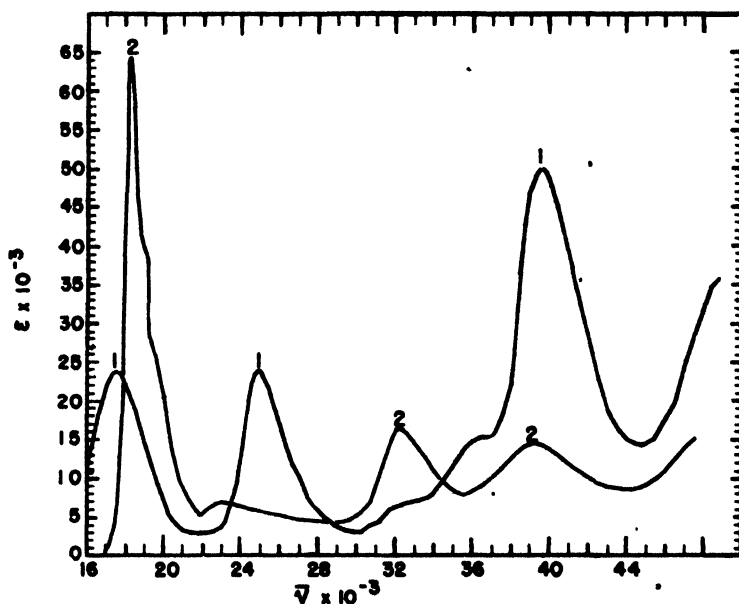


FIG. 7. Absorption curve of Ruhemann's purple. Curve 1, at pH 10.6; Curve 2, in concentrated H_2SO_4 , sp. gr. 1.80.

oxazine derivative. It is similar to the curve for phenoxazine shown by Lewis and Bigeleisen (13). The proposed formula is shown in VI. Oscillation of an electron between the atoms of the nitrogen and oxygen bridge would be the main resonance in an oxazine-free radical, which would account for the following facts. (1) The absorption band in the visible region, maximum at $549 \text{ m}\mu$, is characteristic of oxazine-free radicals, $\lambda = 520$ to $560 \text{ m}\mu$, according to Michaelis, Granick, and Schubert (14). The band is narrow and is about 3 times as intense as in the case of the sodium salt of diketohydrindamine-diketohydrindylidene in water. (2) The color is red in solutions containing 200 mg. of the sodium salt per liter of concentrated H_2SO_4 , but is violet in dilute solution, 4 mg. per liter. Because

it varies with concentration, ϵ in Fig. 7 is arbitrary. For example, at $\lambda = 572 \text{ m}\mu$, the value of ϵ for the concentrated solution is 3.8, for the dilute solution 4.8. (3) The color fades slowly in concentrated H_2SO_4 , sp. gr.

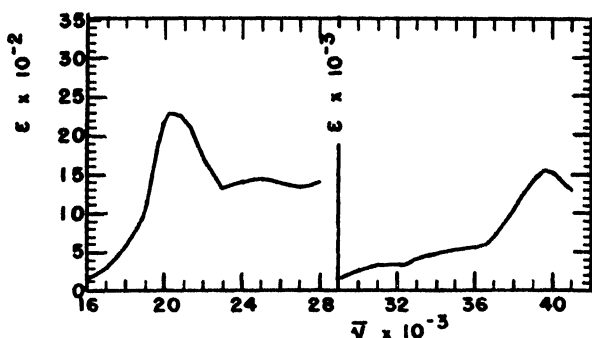
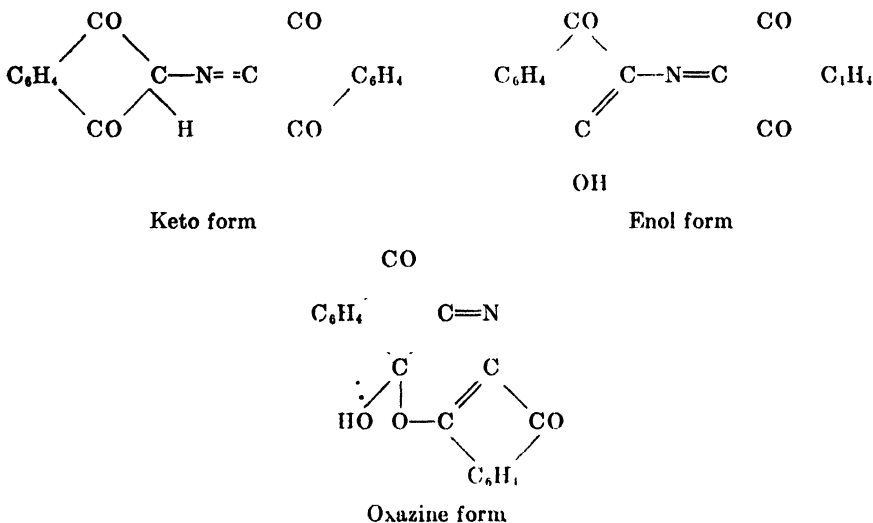


FIG. 8. Absorption curves for diketohydrindamine-diketohydrindylidene· $2\text{H}_2\text{O}$ in chloroform, which is the same for benzene as far as it can be measured.

1.80, the optical density at $\lambda = 549 \text{ m}\mu$ in 4 days at 25° declining to 0.7 of the initial value; for sp. gr. 1.52 there is only a flash of color on dissolving the sodium salt of diketohydrindamine-diketohydrindylidene. In gla-



(VI) Diketohydrindamine-diketohydrindylidene

cial acetic acid fading is rapid, but the spectrum was seen to be similar in contour to that in H_2SO_4 . The instability in acetic acid is greater than that reported for phenoxazine (14), perhaps because the proposed oxazine is less symmetrical in electron pattern than phenoxazine. No

color forms in concentrated HCl, sp. gr. 1.19. (4) Ruhemann's purple reforms when ice-cold 3 N NaOH neutralizes the red H_2SO_4 solution.

Diketohydrindamine-Diketohydrindylidene· $2\text{H}_2\text{O}$ (Fig. 8; Table II)—The band in the visible region is less intense than those of Ruhemann's purple

TABLE II

Some Parameters of Absorption Spectra of Some Bisindane Derivatives of Ninhydrin at 25° in Various Solvents

$\epsilon_{\text{max.}} \times 10^{-3}$	$\lambda_{\text{max.}}$	Solvent	Reference
Sodium salt of diketohydrindamine-diketohydrindylidene (dark green-purple)			
	$m\mu$		
	570	Aqueous propanol	(4)
24.5	570	" pH 10.6	Fig. 7
24.5	400	" " 10.6	" 7
50.0	252	" " 10.6	" 7
(18)*	580	Ethanol	
(21)	410	"	
(16)	585	Acetone, pyridine	
(26)	420	" "	
64.3†	549	Concentrated H_2SO_4 , sp.gr. 1.80	Fig. 7
6.9	435	" " " " 1.80	" 7
16.4	310	" " " " 1.80	" 7
14.5	254	" " " " 1.80	" 7
Ammonium salt of diketohydrindamine-diketohydrindylidene (dark red)			
	500	Glacial acetic acid	(10)
Diketohydrindamine-diketohydrindylidene (?) (dark red)			
	503	Glacial acetic acid	(10)
Diketohydrindamine-diketohydrindylidene· $2\text{H}_2\text{O}$ (light red)			
(2.3)	490	Chloroform, benzene	Fig. 8
(1.9)	400	" "	" 8
(15.5)	252	"	" 8

* The figures in parentheses are approximate, because of fading.

† Arbitrary values of $\epsilon_{\text{max.}}$ (see the text).

or of the oxazine. The band at 252 $m\mu$ is as intense as those of the oxazine. The contour of the curve suggests the oxazine. On extracting the benzene solution with dilute aqueous NaOH at pH 10, Ruhemann's purple forms in the aqueous phase.

In conclusion, three formulae for diketohydrindamine-diketohydrindylidene are noted in structure VI. Resonance involving the enol and oxa-

zine forms in the solid state may be represented in the absorption curve. Fading in the chloroform or benzene solution may indicate a change to the keto form. Pure unhydrated diketohydrindamine-diketohydrindylidene has never been isolated. Ruhemann (1), in reporting on "diketohydrindamine-diketohydrindylidene," noted that its KOH or acid solution rapidly turned yellow, but the purple color formed in dilute ammonium hydroxide. The only pertinent analytical result published by him was for N, found 4.88 per cent; calculated 4.62. A dark red product from benzene extraction of crude Ruhemann's purple had an absorption spectrum in benzene, $\lambda_{\text{max.}} = 500$, more symmetrical than that shown in Fig. 7 and similar to that reported by Purvis (10). The aqueous phase was yellow, indicating no formation of Ruhemann's purple.

I am grateful to Dr. A. Elek of Los Angeles and to Dr. W. Colburn of Chicago for most of the elementary analyses and wish to acknowledge the technical assistance of the Misses Pearl Anderson, Florence Flesch, and Loretta Davis at various times in this investigation.

SUMMARY

1. The absorption spectra are reported for ninhydrin, triketohydrindene, the quinoxaline derivative, ninhydrin ureide and guanide, the dimethyldihydroresorcinol derivative, Ruhemann's purple, and diketohydrindamine-diketohydrindylidene formed in the reaction of ninhydrin with amino acids, proteins, and primary amines generally.

2. Ruhemann's purple is indandione-2-*N*-2'-indanone enolate.

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ON THE MECHANISM OF THE REACTION OF NINHYDRIN WITH α -AMINO ACIDS

II. A SPECTROPHOTOMETRIC STUDY OF HYDRINDANTIN REACTIONS*

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Our purpose is to show by means of a quantitative method for determination of hydrindantin derivatives that previous concepts of the mechanism of the reaction of ninhydrin with amino acids are inadequate or erroneous, and to show facts supporting a new concept..

The main facts are as follows: (a) Hydrindantin forms when amino acids react with ninhydrin (Ruhemann (1); Abderhalden (2)); (b) it reacts with ammonium salts to give Ruhemann's purple ((1); Harding and Warneford (3); Harding and MacLean (4)); (c) ammonia is formed by ninhydrin deamination of amino acids ((1); MacFadyen (5)); (d) hydrindantin in dilute alkaline solution gives a red color, in concentrated alkaline solution a blue or purple color ((1); Retinger (6)). All these facts have been confirmed by us. The reaction of proline or hydroxyproline with ninhydrin is a special case, giving no purple color (Grassmann and von Arnim (7)), and no ammonia (5).

There are three main concepts according to Ruhemann (1), Retinger (6), and Harding *et al.* (3, 4). In the first the amino acid is oxidatively deaminized by ninhydrin, which is reduced to diketohydrindol; diketohydrindol and ninhydrin condense to form hydrindantin, which then combines with ammonia to give Ruhemann's purple. In the second, 2 moles of amino acid combine with hydrindantin and the compound splits into two identical nitrogen-free, purple-colored free radicals, analogously to the presumed compound formed from inorganic cations and hydrindantin in strongly alkaline solution. In the third, amino acids are distinguished from amines and ammonia because the former react faster chromogenically with ninhydrin. Amino acids decompose independently of ninhydrin into a glyoxal and ammonia; the glyoxal reduces ninhydrin to diketohydrindol and is oxidized to the corresponding α -keto acid; ammonia combines with diketohydrindol to form diketohydrindamine, and diketohydrindamine condenses with ninhydrin to form Ruhemann's purple. The sequence of events is the same in the case of amines and ammonia

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except that a glyoxal cannot come from their decomposition but only from the change of ninhydrin into *o*-carboxyphenylglyoxal.

Ruhemann's concept fails to account for the more rapid chromogenic reaction of amino acids with ninhydrin (3, 4), which we have confirmed, and also with hydrindantin, a new fact. Retinger's concept is not in accord with the marked difference in adsorption spectra between Ruhemann's purple (MacFadyen (8)) and hydrindantin in strongly alkaline solution, as shown herein. The concept of Harding *et al.* is inconsistent with the following facts: (a) a negligible amount of ammonia is evolved from amino acids in the absence of ninhydrin under the conditions in which ninhydrin causes evolution of CO₂, purple color, and NH₃ (5); (b) the source of CO₂ cannot be the α -keto acid corresponding to the amino acid, because evolution from keto acids is much slower than from amino acids, according to Van Slyke, Dillon, MacFadyen, and Hamilton (9); amino acids react faster than NH₃ with hydrindantin, as shown herein. With respect to ammonium salts the concept is adequate: hydrindantin formation from ninhydrin alone in aqueous solution at a pH as low as 5, and hydrindantin cleavage into diketohydrindol and ninhydrin at a pH above 4, can be shown by our method.

Our spectrophotometric method is quantitative in contrast to previous ones (1, 3, 4), because exclusion of oxygen during the reactions prevents fading of the red and blue colors of hydrindantin. Control of the oxygen content and of the pH provides for a clear distinction between hydrindantin reactions and those of ninhydrin. These controls are necessary, for, on the one hand, oxidation of hydrindantin yields 2 moles of ninhydrin, and, on the other hand, ninhydrin at certain pH values can form hydrindantin by way of *o*-carboxyphenylglyoxal.

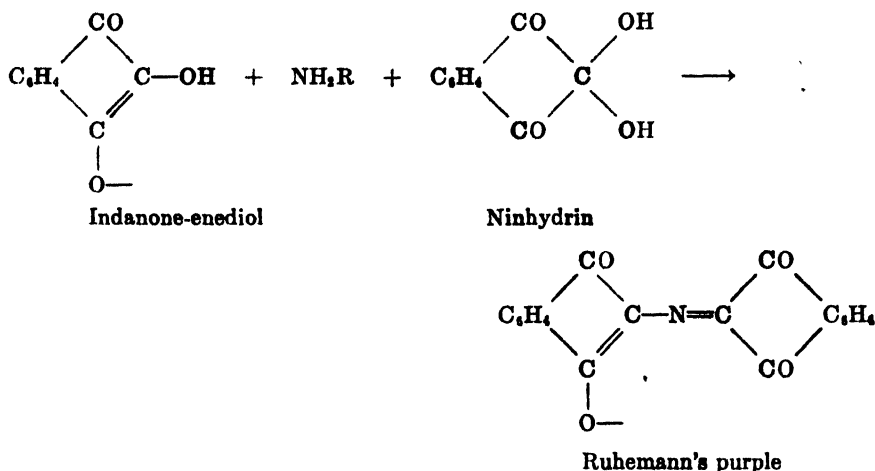
The probable structure of the red-colored derivative of hydrindantin is indanone-enediol and not, as Ruhemann (10) believed, the monovalent salt of intact hydrindantin. In the reaction of amino acids and hydrindantin, 1 mole of indanone-enediol is used up for each mole of Ruhemann's purple formed. The reaction with amines summarized in formulae I could be either a simultaneous or a sequential condensation with indanone-enediol and ninhydrin. The alternatives will be considered in a separate paper on the order of reaction, which the present method has made possible.

Apparatus—

1. Spectrophotometers. The Beckman model DU instrument, with quartz prism, and the Coleman clinical instrument, model 6, were used.

2. Cuvettes and reaction vessels. For the Beckman instrument silica for the measurement of ultraviolet absorption, Corex for visible light. The length of the light path was 1 cm. For the Coleman instrument,

Hamilton vessels (11) were calibrated for length of the light path, which averaged 1.89 cm., and were used as reaction vessels as well.



(I)

Methods

Preparation of Hydrindantin—A solution of 0.5 gm. of ascorbic acid and 1 gm. of ninhydrin in 200 ml. of McIlvaine's buffer (0.1 M) at pH 3 was heated to 90° and the crystals allowed to settle at room temperature. Recrystallization from hot acetone yielded 350 mg. of colorless anhydrous hydrindantin. The purity of the product was checked by elementary analysis and by its melting point (see Abderhalden (2)).

Preparation of Oxygen-Free Reaction Solutions—Since at room temperature water does not dissolve hydrindantin, it was dissolved in acetone in a concentration of 1 mg. per ml. 1 ml. was delivered to each Hamilton vessel and dried by passing a stream of nitrogen above, not in, the solution. 10 ml. of buffer solution, either alone as a control or containing another reagent under test, oxygen-free after a stream of nitrogen was passed through it for 5 minutes, were delivered to each vessel, which was lubricated and quickly closed so as to be air-tight. The gas was then removed by suction from a motor pump until the pressure was constant at about 2 mm. of Hg. Each vessel in the control and test group was again made air-tight and was immersed upright in a frame in a boiling, distilled water bath for a known time interval.

Spectrophotometric Readings—The optical density was recorded at wavelengths of 490 and 570 mμ in the Coleman instrument. Sometimes the measurements were made as quickly as possible after removal from the boiling water bath, in order to record the optical density at a temperature

close to 100°. In such a case, it was found that three measurements could be taken comfortably in from 1 to 2 minutes. For the most part, they were taken at room temperature after cooling in a water bath, and again after passing air through the solutions for 3 minutes.

Absorption Spectra of Red and Blue Colors Derived from Hydrindantin Different from Ruhemann's Purple (Fig. 1)—Solutions of hydrindantin either in Sørensen's NaOH-borate buffer at pH 9.2 or in 0.4 N NaOH were prepared to be oxygen-free in cuvettes for the Beckman instrument.

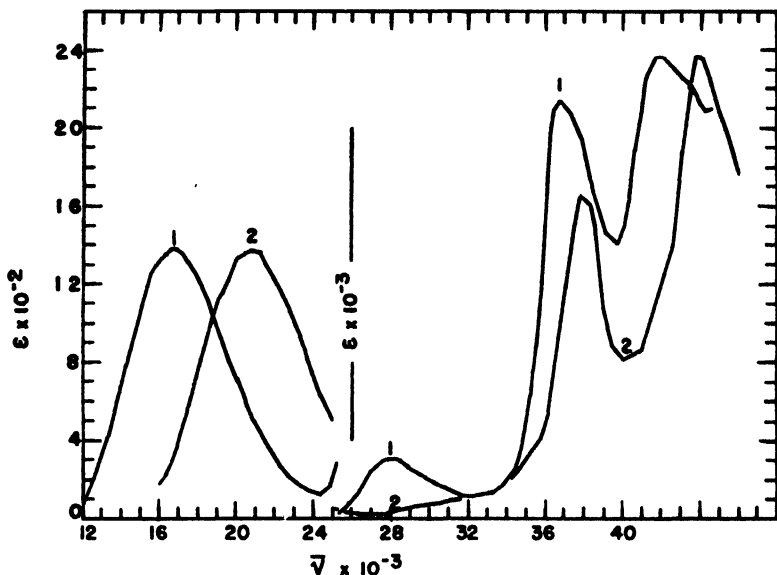


Fig. 1. Absorption curves of the red and blue colors derived from hydrindantin. Curve 1, for the blue color in 0.5 N NaOH; Curve 2, for the red color at pH 9.2. The ordinates refer to the molar absorption coefficients; the abscissae to the wave number per cm.

The visible colors were constant for at least 48 hours, but ultraviolet absorption, without change in position of the maxima, slowly lessened until a constant value was reached at the end of 48 hours. The result is explained by hydrolysis of hydrindantin into diketohydrindol and ninhydrin, followed by irreversible transformation of ninhydrin into either *o*-carboxyphenylglyoxal or *o*-carboxymandelic acid, depending on the pH; either process can be detected by change in the ultraviolet absorption spectrum of ninhydrin (see MacFadyen (8)).

The spectrum, Curve 1, Fig. 1, for the blue color is markedly different from the curve for Ruhemann's purple (8) with respect to position and intensity of maxima. These differences invalidate Retinger's concept (6).

Formation of Ruhemann's Purple from α -Alanine and Hydrindantin Independent of NH_3 Pathway (Table I)—At pH 5.3 at 100° , and anaerobically, the intensity of purple color in the reaction of hydrindantin, 0.3 mM, with the amino acid was 5 times that for ammonium salts in the same concentration, 0.56 mM. Therefore, the concepts of Ruhemann (1) and Harding *et al.* (3, 4), necessitating an NH_3 pathway, are inadequate. The

TABLE I

Formation of Ruhemann's Purple from α -Alanine and Hydrindantin with Disappearance of Hydrindantin Red Color

Reaction time in boiling water bath	Observed optical densities, units $\times 10^2$					Hydrindantin disappearance	Formation† of Ruhemann's purple
	α -Alanine			Present	Absent		
	P_{570}	D_{490}^-	D_{490}^+	R'_{490}	R''_{490}		
	a^*	b	c	$d = b - c$	e	$f = 687(e - d) \times 10^{-3}$	$g = af/f_1 \times 10^{-3}$ (average)
min						mM per l.	mM per l.
10	360	283‡	123	160	175	4.8–13.7	12.1
	360	290	122	168	180		
20	620	365	200	165	187	15.1–20.6	22.7
	640	363	199	164	194		
30	860	441	278	163	205	25.4–31.6	35.5
	880	440	281	159	200		
40	1010	465	324	141	212	39.2–52.9	46.4
	1000	498	338	160	218		
50	1150	492	370	122	232	74.8–77.0	63.5
	1280	541	418	123	234		
60	1290	546	434	112	230	77.0–81.0	76.4
	1360	566	450	116	228		

* For explanation of P_{570} , R_{490} , computation of the factor 687, and for f_1 and f_2 see the text.

† For $(\text{NH}_4)_2\text{SO}_4$ the results, in chronological order, were 2.2, 5.0, 5.7, 9.5, 14.3, and 15.7.

‡ The third figure was estimated by interpolation.

experimental details are discussed below in connection with the mechanism of the reaction.

Hydrindantin Red Color Disappears As Ruhemann's Purple is Formed, Mole for Mole (Table I)—Difficulties of quantitative estimation of concentrations of Ruhemann's purple and the red color from hydrindantin, together in reaction mixtures, were obviated in the following ways.

Ruhemann's purple is decolorized at pH 5.3, even under anaerobic conditions, when its solutions are heated to boiling, but not significantly at room temperature. Therefore, estimates of the amount formed in a given

time interval, in contrast to the amount present, required measurements of the intensity of the purple color remaining in solution of known amounts of the sodium salt of diketohydrindamine-diketohydrindylidene (8) under the conditions of the α -alanine reaction with hydrindantin including all reagents except α -alanine. Such tests provided us with factors, f_1 , by which the optical density at $\lambda = 570 \text{ m}\mu$ observed at a given time interval of boiling was converted into the initial optical density before heating. Furthermore, while it is true that Ruhemann's purple obeys Beer's law (8), it was necessary to correct for spectrophotometric conditions of the Hamilton vessels and the Coleman instrument with respect to deviations from Beer's law, but not in the case of the red color. For this purpose the sodium salt of diketohydrindamine-diketohydrindylidene was dissolved in buffer at pH 9, and the optical densities at varied concentration determined in the Hamilton vessels in the Coleman instrument. From these results factors, f_2 , were computed which when multiplied by the optical density at $\lambda = 570 \text{ m}\mu$, in so far as it is referable only to Ruhemann's purple, yielded the concentration of the sodium salt in micromoles per liter.

Application of the factors, depending on a clear distinction between the purple color due to the sodium salt and the red color due to hydrindantin, was made by taking advantage of two facts. Whereas Ruhemann's purple is stable when oxygenated at pH 10 to 11 at room temperature, the red color is discharged in 3 minutes. Therefore, the optical density after oxygenation, measured at $\lambda = 570 \text{ m}\mu$, D_{570}^{+O} , when corrected in the usual manner for variation in length of the light path and translucency from vessel to vessel, is representative of Ruhemann's purple and is denoted as P_{570} , whereas the optical density difference due to oxygenation, measured at $\lambda = 490 \text{ m}\mu$, $D_{490}^{+O} - D_{490}^{-O}$, when similarly corrected, is representative of the hydrindantin red color and is denoted by R_{490} .

The experiments were carried out with 0.5 mg. of α -alanine (or 0.37 mg. of $(\text{NH}_4)_2\text{SO}_4$) and 1 mg. of hydrindantin in 10 ml. of 0.1 M acetate buffer at pH 5.3. The solutions, in duplicate, were heated to boiling, anaerobically, for a given time interval of 10, 20, 30, 40, 50, or 60 minutes. Then they were cooled in ice water for 4 minutes and brought to room temperature at 27° , about 25 minutes later. The optical densities in the absence of oxygen, D^{-O} , were recorded. The vessels were opened, the pH of the solutions changed to 10 to 11 by adding about 0.02 ml. of 40 per cent NaOH, and the red color was discharged by bubbling air through the solutions for 3 minutes. Then the optical densities, D^{+O} , were recorded. The observed data were converted to micromolar concentrations (X) of substance responsible for the red or purple colors as follows: In the case of the red color, $(X) = R_{490}/(\epsilon \times 10^{-6} \times l \times \alpha)$, where ϵ is the molar ab-

sorption coefficient at $\lambda = 490 \text{ m}\mu$ and at pH 9.2, assuming complete hydrolysis of hydrindantin into diketohydrindol, l is the length of the light path in cm., and α is the ratio of optical density at pH 5.3 to that at pH 9.2. The numerical values were 1400, 1.89, and 0.55 respectively. The equation simplifies to $(X) = 687R_{490}$. In the case of the purple color, $(X) = P_{570} \times f_1 \times f_2$, previously described. For the time intervals 10, 20, 30, 40, 50, and 60 minutes the numerical values of f_1 were 1.31, 1.44, 1.58, 1.74, 1.91, and 2.10, respectively, and for f_2 were 24.8, 25.2, 25.8, 26.5, 27.4, and 27.5, respectively.

The disappearance of red color associated with formation of Ruhemann's

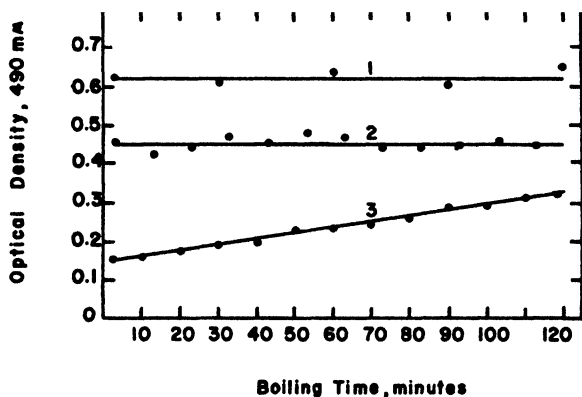


FIG. 2. Hydrolysis of hydrindantin (0.31 mm) at pH 5.3 in boiling aqueous anaerobic solution with and without dimethyldihydroresorcinol. Curve 1, optical density at 100°, with or without dimethyldihydroresorcinol; Curve 2, optical density after cooling to 25°, in the presence of dimethyldihydroresorcinol; Curve 3, optical density after cooling to 25°, without dimethyldihydroresorcinol.

purple was calculated from R'_{490} , obtained when the reaction mixture contained α -alanine, and from R''_{490} , obtained when all reagents were present except α -alanine. The disappearance, in terms of micromoles of hydrindantin per liter, $= 687(R'' - R')_{490}$.

In the case of α -alanine, the results show that the disappearance of hydrindantin red color is proportional to the formation of Ruhemann's purple, within the limits of error of the method.

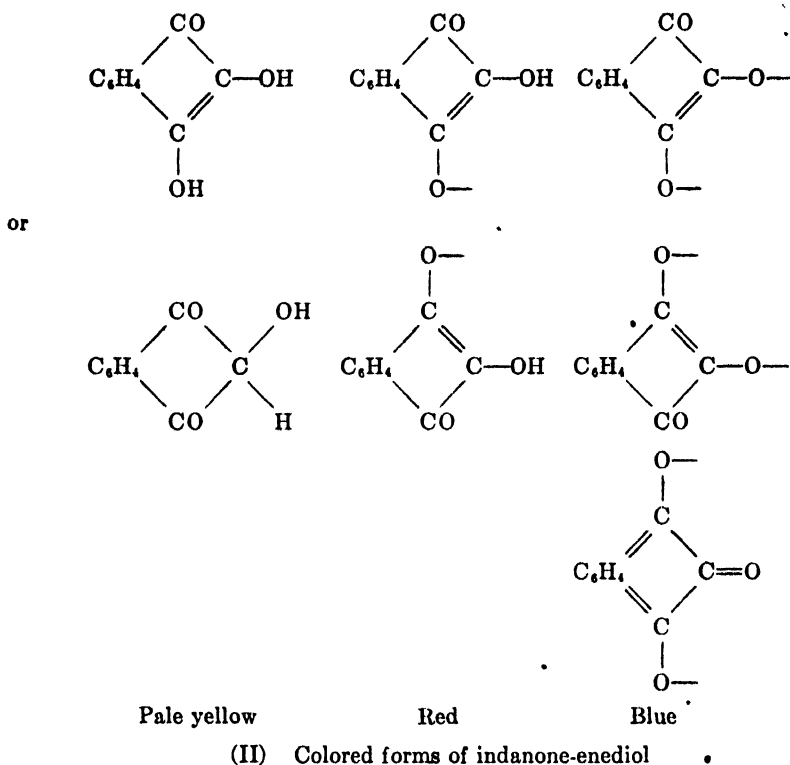
Red and Blue Colors from Hydrindantin Due to Diketohydrindol (Fig. 2)—Our claim that the red color evolved from hydrindantin, as well as the undisputed blue color (1), is due to diketohydrindol rests on the following facts. (a) The blue color is reversibly changed into the red by acid-base titration under anaerobic conditions, $\text{pK}' = 12.3$ at 25°. When the blue color is formed, the other component of hydrindantin, namely ninhydrin, is changed into *o*-carboxymandelic acid (1) by irreversible internal oxida-

tion-reduction. This change is complete in a few minutes, but the color change is quantitatively reversible for days. Therefore, the change from blue to red does not necessitate the reformation of hydrindantin; the claim (10) that the red color is due to the monovalent anion of hydrindantin is invalid. (b) The same play of colors with change in pH was observed by Hassall (12) in connection with the hydrolysis of acetoxindandione to diketohydrindol, which was identified by adding ninhydrin to acidified solutions from which hydrindantin was obtained. (c) In acid solutions of hydrindantin dimethyldihydroresorcinol accelerates the formation of the red color, which attains a constant intensity for a given concentration of hydrindantin. The explanation offered is hydrolysis of hydrindantin into the red color and ninhydrin, accelerated by combination with the resorcinol (see Fig. 2). The resorcinol combines with ninhydrin (8). The compound, inert to oxygenation, can be detected spectrophotometrically in the solutions after oxygenation. (d) Above pH 7, there is no difference in red color caused by dimethyldihydroresorcinol or by cooling the solutions from 100° to room temperature. If a red alkaline solution is acidified to pH 5.3, anaerobically, the color fades as hydrindantin is precipitated. The data could be interpreted, as Ruhemann concluded (10), to show that intact hydrindantin is responsible for the red color. However, at a pH, temperature, and time interval (pH 10, 25°, 24 hours) insuring complete irreversible transformation of ninhydrin to *o*-carboxyphenylglyoxal, acidification no longer causes reformation of hydrindantin, the fading of the red color being what would be expected from its titration curve, $pK' = 5.2$. In this case, addition of ninhydrin causes and is necessary for precipitation of hydrindantin. (e) By careful adjustment of the concentration of added hydrosulfite, the intensity of the red color from hydrindantin can be doubled.

Having shown that the red and blue colors of hydrindantin solutions are derivatives of diketohydrindol readily convertible under anaerobic conditions one to the other and to diketohydrindol, simply by change in pH ($pK' = 5.2$ and 12.3), we conclude that the colors are due to the anions of diketohydrindol. Enolization of diketohydrindol would allow for two ionizable groups. Therefore, the red color is attributable to the monovalent anion, the blue color to the divalent anion. The ease of oxidation on exposure to air is consistent with the indene structure formable by enolization. The enediol formulae shown in II provide for resonance which would explain the difference in color and the chemical behavior of the substances.

On reduction with hydrosulfite the red and blue colors disappear but careful oxygenation restores their original intensity. On the other hand,

the effect of oxygenation is not reversible in the case of the blue color and is only reversible in the case of the red color if ninhydrin is present in solution. Irreversibility is explained by oxidation of the red color to *o*-carboxyphenylglyoxal and of the blue color to *o*-carboxymandelic acid.



Mechanism of Reaction of α -Alanine with Hydrindantin—The disappearance of the red color in this reaction may now be reconsidered. It is ascribed to conversion of indanone-enediol into Ruhemann's purple, the anion of diketohydrindamine-diketohydrindylidene. The alternative, reduction of Ruhemann's purple ($\text{C}_{18}\text{H}_8\text{O}_4\text{N}^-$), which would become colorless while the enediol was oxidized to colorless ninhydrin, is untenable, because the chromogenic reaction of α -alanine is faster with 1 mole of hydrindantin ($\text{C}_{18}\text{H}_{10}\text{O}_6$) than with 2 moles of ninhydrin ($\text{C}_9\text{H}_6\text{O}_4$). The non-enolic component of Ruhemann's purple must be supplied by ninhydrin, also from hydrolysis of hydrindantin, for only 1 mole of indanone-enediol is used up for each mole of Ruhemann's purple formed. The details of the mechanism, summarized in formula I, will be discussed in a paper on the order of the reactions of amines with hydrindantin and ninhydrin.

SUMMARY

1. The red and blue colors formed by dissolving hydrindantin in alkaline solution have been studied spectrophotometrically under controlled conditions of oxygen content, pH, and temperature. The results indicate that the red color represents the monovalent anion of indanone-enediol and the blue color represents the divalent anion.

2. At pH 5.3, the sodium salt of diketohydrindamine-diketohydrindylidene is formed in the reaction of α -alanine with hydrindantin but not by way of intermediate ammonia formation. Indanone-enediol is used up in the reaction mole for mole of Ruhemann's purple formed.

3. Of previous concepts of the mechanism of the reaction of amino acids and other amines with ninhydrin, only that proposed with respect to ammonium salts by Harding, Warneford, and MacLean (3, 4) is supported by the present results.

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AMINO ACID COMPOSITION OF EGG PROTEINS

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The amino acid composition of a number of egg proteins under investigation in this Laboratory has been determined by microbiological and other methods. The results of these determinations, a description of the microbiological methods, and analyses of reference proteins are reported here.

Materials and Methods

Lysozyme prepared from egg white by the method of Alderton and Fevold (1) was supplied by these authors. The material had been recrystallized five times from sodium bicarbonate (pH 8), once from 5 per cent sodium chloride (pH 4.4), dialyzed, and reprecipitated near the isoelectric point (pH 10.7). Ovomuroid, prepared by the method of Line-weaver and Murray (2), vitellin, prepared by the method of Alderton and Fevold (3), and phosvitin, prepared by the method of Mecham and Olcott (4), were supplied by the respective authors. Conalbumin (5) was obtained from H. F. Deutsch of the University of Wisconsin, who found it to be about 95 per cent pure electrophoretically. Tests in this Laboratory showed it to be of similar iron-binding capacity as preparations made according to Alderton *et al.* (6). Avidin was prepared by a method yet to be described. It was of slightly higher activity than preparations described previously (1 mg. of avidin bound 8 γ of biotin), and is believed to be about 90 per cent pure.

β -Lactoglobulin and chymotrypsinogen were obtained through the courtesy of E. F. Jansen. β -Lactoglobulin was prepared by the method of Palmer (7) and recrystallized four times by solution in dilute sodium chloride followed by dialysis at the isoelectric point (pH 5.2) of the protein. The final product was lyophilized. Chymotrypsinogen, purchased from the Plaut Research Laboratory, Lehn and Fink Products Corporation, Bloomfield, New Jersey, was recrystallized (8) seven times and exhaustively dialyzed and then lyophilized. Egg albumin was supplied by W. H. Ward. It was recrystallized three times after isolation by the method

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of Kekwick and Cannan (9). Bovine serum albumin was crystalline material, Lot 46, from Armour and Company.

Total nitrogen was determined by the micro-Kjeldahl method with mercuric oxide as the catalyst. *Amino nitrogen* was determined in the manometric Van Slyke apparatus, either with a 15 minute reaction time, or for increasing time periods from 15 minutes to 2 hours, the true amino nitrogen being obtained by extrapolation. Reproducibility was not very good in 1 and 2 hour runs. Triplicate analyses were averaged. Results obtained by extrapolation to zero time were from 5 to 11 per cent lower than those of the 15 minute reaction period. End-group calculations based on these values agreed with those obtained with other techniques, indicating that the extrapolated was the correct value. *Amide nitrogen* was determined by autoclaving in 1.2 N sulfuric acid at 120° for 40 minutes and distilling ammonia from the hydrolysate made alkaline with a phosphate buffer of pH 7.4. *Sulfur* was determined by the sodium peroxide fusion method. *Moisture* was determined by drying to constant weight at 102°. All results are reported on a moisture-free basis. *Tryptophan* was determined on unhydrolyzed samples by the method of Spies and Chambers (10), with a 12 hour reaction period and 0.05 ml. of 0.1 per cent nitrite.¹ *Acid* and *basic groups* were determined by the dye method (11). Because of the high isoelectric point of lysozyme, acid groups could be determined only on less basic derivatives (12) of this protein. The basic groups of ovomucoid could not be determined by this method, since the dye-protein complex did not precipitate. *Cystine* was determined by the Sullivan method after hydrolysis with a mixture of concentrated hydrochloric and 95 per cent formic acid (1 ml. and 0.8 ml., respectively, for 10 to 40 mg. of protein). Ovomucoid and conalbumin showed the same cystine content when hydrolyzed instead with 6 N hydrochloric acid for 24 hours, but lysozyme and vitellin gave lower values.

For microbiological assay, the proteins were hydrolyzed by refluxing for 18 hours with 6 N hydrochloric acid (20 ml. per gm. of protein). The hydrolysates were dried *in vacuo* to remove most of the acid, and diluted to prepare a stock solution. Aliquots were neutralized before assay with *Leuconostoc mesenteroides* P-60 (ATCC 8042) (arginine, aspartic acid, glycine, histidine, lysine, phenylalanine, proline, serine, and tyrosine), *Lacto-*

¹ The color given by lysozyme and conalbumin under the test conditions differed from that of the standard (blue, as compared to gray-purple), but good agreement was obtained for widely different amounts of protein. This is in contrast to results obtained with older modifications of the dimethylaminobenzaldehyde method. Alkaline hydrolysis (without the precautions suggested by Spies and Chambers (10)) caused progressive destruction of tryptophan without preventing the variations depending upon assay level observed for the older modifications.

bacillus arabinosus 17-5 (ATCC 8014) (glutamic acid, isoleucine, leucine, methionine, and valine), *Leuconostoc citrovorum* (ATCC 8081) (alanine and serine), and *Streptococcus faecalis* R (ATCC 8043) (threonine). A general basal microbiological assay medium was formulated to contain concentrations of the amino acids high relative to the requirements of the test bacteria and also high relative to the amounts of the amino acids normally introduced in the protein hydrolysates. This medium (double strength) contained the following: (in gm. per liter) glucose 40, sodium acetate (trihydrate) 4, sodium succinate 20, potassium phosphate (monobasic) 2, ammonium chloride 10, magnesium sulfate (heptahydrate) 0.4, ferrous sulfate (heptahydrate) 0.02, manganous sulfate (tetrahydrate) 0.08, DL-alanine 4, L-arginine 0.6, L-asparagine 2, DL-aspartic acid 0.4, L-cystine 0.8, L-glutamic acid 2, glycine 2, L-histidine 0.4, hydroxy-L-proline 0.2, DL-isoleucine 0.8, L-leucine 0.6, L-lysine 0.6, DL-methionine 0.6, DL-norleucine 0.2, DL-norvaline 0.2, DL-phenylalanine 0.6, L-proline 0.6, DL-serine 0.6, DL-threonine 1.0, DL-tryptophan 0.4, L-tyrosine 0.4, DL-valine 0.8, adenine sulfate 0.04, guanine hydrochloride (dihydrate) 0.04, uracil 0.04, xanthine 0.04; (in mg. per liter) thiamine hydrochloride 4, pyridoxine hydrochloride 2, pyridoxamine dihydrochloride 2, calcium pantothenate 4, riboflavin 4, nicotinic acid 2, nicotinamide 2, *p*-aminobenzoic acid 0.02, biotin 0.02, pteroylglutamic acid 0.02. The medium had to be buffered further for *S. faecalis* as follows: (in gm. per liter) potassium phosphate (dibasic, trihydrate) 20, sodium citrate 40. The medium of Steele *et al.* (13) was used for *L. citrovorum*. For serine assays both *L. citrovorum* and *L. mesenteroides* were used with the latter medium, and the closely agreeing results were combined. The medium described above gave very discordant results for serine with *L. mesenteroides*.

Stock cultures were maintained on Difco tomato juice agar. The inoculum medium differed from the basal medium mentioned above in the substitution of 5 gm. of acid-hydrolyzed casein per liter of diluted medium for all of the amino acids except tryptophan and cystine. • 1 drop of a 7-fold dilution of a saline resuspension of a 24 hour culture was used per tube. Cultures were grown in a 4 ml. volume at 35° for approximately 64 hours. Normal net blank titrations (inoculated blank less the uninoculated blank) did not exceed 1.8 ml. of 0.05 N NaOH per 4 ml. of culture. Normal net maximum growth controls were approximately 12, 11, 14, and 14 ml. of 0.05 N NaOH for *L. mesenteroides*, *L. citrovorum*, *L. arabinosus*, and *S. faecalis*, respectively. Dosage-response curves were sigmoidal with glutamic acid and proline, and to a lesser degree with glycine, isoleucine, and threonine.

L-Amino acids were used as standards except for the cases in which lack

of pure L acids necessitated the use of DL acids (alanine, isoleucine, serine, threonine, and valine).² A number of different preparations were tested in most cases. D-Alanine is assumed to be completely active, while the D isomers of isoleucine, serine, threonine, and valine are assumed to be completely inactive. The desirability of using the L isomer as a standard for non-racemized hydrolysates is emphasized by the number of cases for which the D isomer has been found to have significant activity. We found DL-alanine to be 105 per cent as active as L-alanine (possibly not pure) for *L. citrovorum*, DL-aspartic acid 80 per cent as active as L-aspartic acid for *L. mesenteroides*, DL-glutamic acid 60 per cent as active as L-glutamic acid for *L. arabinosus*, DL-leucine 58 per cent as active as L-leucine for *L. arabinosus*, and DL-methionine 116 per cent as active as L-methionine for *L. arabinosus*. All but the latter observation are in agreement with previous findings. The unexpectedly high activity of DL-methionine is probably related to the inclusion of 1 mg. of pyridoxamine dihydrochloride per liter of medium; Camien and Dunn (15) have recently reported that one-tenth this concentration would permit complete utilization of D-methionine by *L. arabinosus*. The finding was confirmed with pure D- and L-methionine prepared by Spies (16); D-methionine proved to be 126 per cent as active as L-methionine for *L. arabinosus* but less than 1 per cent as active for *L. citrovorum*.

Microbiological assays of β -lactoglobulin, bovine serum albumin, chymotrypsinogen, and egg albumin made to check the reliability of the methods are given in Table I. The results may be compared most conveniently with the analyses compiled by Tristram (18). The alanine values run 10 to 20 per cent lower than values in the literature. The other values for β -lactoglobulin, bovine serum albumin, and egg albumin check within ± 5 per cent with the most consistent values reported for non-microbiological methods, except for histidine, leucine, and lysine in serum albumin and valine in β -lactoglobulin which check within ± 10 per cent, and isoleucine in β -lactoglobulin and valine in egg albumin which run very high. The analyses of chymotrypsinogen deviate in many instances from the published values (19).

Paper chromatography (20) was employed to show the presence of methionine and proline and the absence of hydroxyproline in protein hydrolysates. The ascending solvents were either a mixture of *n*-butanol (200 ml.), glacial acetic acid (30 ml.), and water (75 ml.), or water-saturated

² The senior author of this paper erred in reporting values for sheep lactogenic hormone (14) based on commercial preparations of L-isoleucine, L-threonine, and L-valine which were subsequently found to be impure. Revised values, based on the standards used in the present work, are as follows: isoleucine 5.6, threonine 4.1, and valine 4.6 gm. per 100 gm. of dry protein,

collidine. Methionine was detected on the paper (Whatman No. 1) by iodoplatinate (Winegard *et al.* (21)), proline by spraying with isatin (0.2 per cent) (22) and subsequent heating (10 minutes at 90°). A series of varicolored spots on a yellow background was obtained with both acid and alkaline hydrolysates of lysozyme, as well as with a mixture of amino acids resembling the composition of lysozyme. In each case only one blue spot was obtained and this had an R_f value similar to that of proline. Hydroxyproline gave a hardly perceptible spot with isatin; therefore nin-

TABLE I
Amino Acid Composition of Reference Proteins

Results in gm. of amino acid in hydrolysate from 100 gm. of dry protein.

Constituent	β -Lactoglobulin	Bovine serum albumin	Egg albumin	Chymotrypsinogen
Alanine	5.8	5.0	5.7	5.8
Arginine	2.88	5.7	5.9	2.73
Aspartic acid	11.3	10.2	9.2	10.9
Glutamic "	18.4	16.2	15.7	7.4
Glycine	1.38	1.75	3.20	6.3
Histidine	1.66	3.68	2.41	1.16
Isoleucine	7.3	2.65	7.1	4.9
Leucine	15.2	12.0	9.9	8.9
Lysine	11.2	11.5	6.4	7.7
Methionine	3.12	0.85	5.4	1.11
Phenylalanine	3.63	6.3	7.5	3.77
Proline	5.0	4.6	3.8	3.8
Serine*	3.8	4.0	8.5	10.9
Threonine	5.2	5.7	4.0	10.7
Tyrosine	3.87	4.9	3.75	2.69
Valine	6.2	6.1	8.8	10.3
N, %	15.6	16.1	15.8	16.4

* Assay values increased by 10 per cent to compensate for destruction during acid hydrolysis (17).

hydrin was chosen for its detection. Small amounts of added hydroxyproline could not be detected in two-dimensional chromatographs because of overshadowing by nearby spots of amino acids present in large excess. Therefore, the following technique was adopted: 0.2 ml. of hydrolysate (equivalent to 20 mg. of protein) was spread in a narrow band 1.25 inches from the long edge of an 18 × 22.5 inch sheet of filter paper. The paper was formed into a cylinder (with the hydrolysate band near the bottom), which was humidified and set in a covered jar containing a thin layer of water-saturated collidine and a dish of water. The solvent was allowed to ascend about 30 cm.; then the paper was removed to a hood and allowed

to dry. The paper was then cut into strips 1 cm. wide ($\Delta R_f = 0.033$ approximately), parallel to the original hydrolysate band, in the region about the hydroxyproline band ($R_f = 0.34$ approximately). The strips were eluted with water and the eluates were concentrated to 0.1 ml. Spots of the eluates were then chromatographed one-dimensionally with *o*-cresol and with phenol. 1 mg. of hydroxyproline added to the hydrolysate from 100 mg. of lysozyme was recognized very readily.

Results

The amino acid content of several egg proteins is given in Table II. Cysteine ($-SH$) was not detected in any of these by the nitroprusside test after denaturation with guanidine salts. Hydroxyproline was not detected by paper chromatography in lysozyme, conalbumin, ovomucoid, vitellin, or egg albumin; avidin and phosvitin were not tested.

The analysis of *lysozyme* is of particular interest because of its purity and known low molecular weight. The results are in approximate agreement with the most recent data reported by Fromageot and Privat de Garilhe (23). Alanine, glycine, histidine, leucine, lysine, and threonine values check within 5 per cent, and serine, proline, methionine, and tyrosine values agree within 10 per cent. Some of the greater discrepancies, as for aspartic acid, glutamic acid, and arginine, may be related to the fact that their tests were made on fractions of lysozyme hydrolysates.³ Our cystine value is lower than theirs (8.0 per cent), but in view of the lability of cystine under various conditions of hydrolysis, the higher value is probably correct.⁴

In view of the suggested absence of methionine and proline (24), we have confirmed our microbiological tests by identifying methionine and proline by specific color tests (21, 22) on paper chromatograms of unfractionated lysozyme hydrolysates. Both amino acids showed R_f values in two solvents similar to those of the corresponding spots of the acid or alkaline hydrolysate (Table III). Identical R_f values were obtained when an amino acid mixture resembling lysozyme in its composition was compared with the protein hydrolysate. The intensity or size of the spots suggested lower concentrations for both amino acids, particularly proline, than were found by microbiological assay.

The calculations summarized in Table IV show that 97 per cent of the

³ Recent microbiological assays have led Dr. Fromageot to revise some of these values, leading to better agreement with our values (personal communication).

⁴ Independent evidence that the higher cystine content found by Fromageot *et al.* (23) is probably correct will be published elsewhere (12). Hydrolysis in the presence of formic acid can in no way be counted on to prevent destruction of cystine, which was found to be quite extensive in certain hydrolysates.

solids and 98.5 per cent of the nitrogen are accounted for, which values would be low to the extent that the alanine value is low. The composi-

TABLE II
Composition of Egg Proteins

Results in gm. per 100 gm. of protein, except for the acid and basic groups which are in equivalents per 10⁴ gm. of protein.

Constituent	Lysozyme	Conalbumin	Ovomucoid*	Avidin	Phosvitin*	Vitellin
Alanine	5.8	4.4	2.3	+	1.5	4.0
Arginine...	12.7	7.6	3.7	6.5	4.8	8.4
Aspartic acid	18.2	13.3	13.0	9.7	4.4	8.1
Cystine.	(6.8)†	3.8	6.7	0.46	0.0 (4)	1.5
Glutamic acid	4.32	11.9	6.5	6.6	3.4	11.0
Glycine.	5.7	5.7	3.8	4.6	1.6	2.8
Histidine	1.04	2.57	2.15	0.96	4.8	3.0
Hydroxyproline	Nil	Nil	Nil			Nil
Isoleucine	5.2	5.0	1.43	5.5	0.5	5.3
Leucine	6.9	8.8	5.1	4.9	1.0	8.6
Lysine ..	5.7	10.0	6.0	6.2	5.9	6.9
Methionine	2.06‡	2.03	0.95	1.41	0.3	2.8
Phenylalanine	3.12	5.7	2.91	5.9	0.6	4.0
Proline	1.40	4.9	2.72	1.64	1.0	4.4
Serine§	6.7	6.3	4.2	4.5	33	11.2
Threonine	5.5	5.9	5.5	10.5	1.4	4.7
Tryptophan.	10.6	3.0	≤ 0.3 (2)	5.4	0.6 (4)	1.1
Tyrosine	3.58	4.6	3.18	0.88	0.1	3.8
Valine...	4.8	8.2	6.0	4.2	1.1	6.2
Amide N	1.71	1.04	1.00	1.3	0.8 (4)	1.29
Free amino N	0.74	1.11	0.68	1.1	0.7 (4)	0.73
Total N	18.6	16.6	13.1	14.8	11.9 (4)	15.7
“ S ..	2.53	1.83	2.2 (2)	0.95	< 0.1 (4)	0.99
“ P.	0.0	0.0	0.0	0.79	9.7 (4)	2.2
Acid groups	(6.7)†	14.1	13.4	8.1	56	18.1
Basic “	13.0	14.0		8.6	•	7.5

* The numbers in parentheses refer to the bibliography.

† Approximate values.

‡ Values for methionine in lysozyme varied from 1.6 to 2.1 by *L. arabinosus*; the average of closely agreeing values obtained with *L. citrovorum* and *L. mesenteroides* is given.

§ Assay values increased by 10 per cent to compensate for destruction during acid hydrolysis (17).

|| Values obtained by extrapolation to zero time.

tion of lysozyme thus appears sufficiently well elucidated to permit some structural interpretations. The 2 residues of methionine found by us and

the 5 residues of cystine found by Fromageot and Privat de Garilhe (23) account quantitatively for the sulfur of the protein. The cystine content (there is no cysteine (12)) would permit the existence of a maximum of six peptide chains per molecule. However, calculations of the terminal amino and carboxyl groups by difference between total basic, acid, and amino groups on the one hand, and the respective amino acids on the other (Table IV), suggest that the actual number is two or one.⁵ Other considerations (12) favor a single peptide chain. The net excess of basic over acid groups (about ten per molecule) is consistent with the basic nature of lysozyme.

TABLE III
R_F Values of Proline and Methionine

Solution chromatographed	Butanol-acetic acid	Collidine
Proline....	0.24	0.14
Amino acid mixture, acid	0.28*	0.13
Lysozyme hydrolysate, acid	0.28	0.13
" alkaline	0.28	
Methionine	0.42	0.29
Amino acid mixture, acid..	0.03, 0.45*	0.03, 0.26*
Lysozyme hydrolysate, acid .	0.03, 0.44	0.03, 0.27
" alkaline	0.40	0.27
Cystine	0.03	0.03

* The chromatogram was not affected if the 5 N hydrochloric acid solution of the amino acid mixture simulating lysozyme was first refluxed or was evaporated directly.

The minimal molecular weight is 14,900 on the basis of 1 histidine residue per molecule. The minimal molecular weight of 14,900 is supported by the results of assays for other amino acids; most of those represented by 2 to 8 residues per molecule approach the expected integral values closely (Table IV) when calculated on the basis of this molecular weight. In agreement with this, a molecular weight of 14,700 can be calculated from the sedimentation velocity and the diffusion coefficient published by Alderton *et al.* (26) and the partial specific volume of 0.720 calculated from the

⁵ The applicability of these techniques for approximate determinations of the number of chain ends in proteins was tested with egg albumin. Only 0.2, 0.3, and -0.3 end-groups per 10⁴ gm. of protein are found by the three modes of calculation, if it is assumed that the -SH group is not free to bind the basic dye and that the phosphate carries only one effective charge. No end-group is detectable with dinitrofluorobenzene (25).

amino acid composition according to the principles⁶ outlined by Cohn and Edsall (28). The molecular weight of 14,500 obtained by light-scattering measurements⁷ is also in close agreement. A slightly lower value of $13,900 \pm 600$ was obtained by Palmer *et al.* (29) from x-ray diffraction measurements, and higher values of 17,500 (26) and 16,600⁸ by osmotic pressure determinations.

The nitrogen and solids of *conalbumin* are accounted for almost quantitatively (Table IV). The similar numbers of acid and basic groups are consistent with the isoelectric point of 6.1 reported by Bain and Deutsch (5). The molecular weight of approximately 87,000 (5) is too large to permit establishing the exact numbers of amino acid residues. The number of peptide chains appears to be approximately nine. The cystine and methionine residues fail to account quantitatively for the sulfur found by direct determination; this may be due to loss of cystine during hydrolysis.

The amino acid contents listed in Table II represent 86 per cent of the total nitrogen (Table IV) of *ovomucoid*. The rest of the nitrogen is partially accounted for by glucosamine. Thus Stacey and Woolley (30) report that a carbohydrate fraction, which makes up 20 per cent of *ovomucoid*, consists of 7 *N*-acetylglucosamine, 3 mannose, and 1 galactose residues. This corresponds to 5.5 per cent of nitrogen in the carbohydrate moiety, or 8.4 per cent of the total nitrogen of the protein. Hewitt (31) found 9.5 per cent glucosamine in *ovomucoid* by a colorimetric test on a hydrochloric acid hydrolysate. This corresponds to 5.7 per cent of the total nitrogen (assuming 13.1 per cent total nitrogen). Only 66 per cent of the total solids is accounted for as amino acids.

The number of moles of amino acid in 28,800 gm. of *ovomucoid* (Table IV) approaches the expected integral values closely for those amino acids present to the extent of 2 to 8 moles (except alanine). These results represent good evidence in support of the molecular weight of 28,800 found by osmotic pressure (2). Cystine and methionine account for 90 per cent of the sulfur found by analysis. The difference between the number of free amino groups and the number of lysine residues suggests the presence of two peptide chains per molecule. However, the thirty-nine acid groups found per molecule are greatly in excess of the number of acidic amino acids reduced by the number of amide groups (Table IV). The previous finding that the amino acids account for almost all the protein nitrogen

⁶ The partial specific volume of the cystine residue was assumed to be 0.63 rather than 0.61 (27).

⁷ N. Halwer, in preparation for publication.

⁸ A. Mohammad, unpublished experiments. The slow dialyzability of lysozyme under the test conditions was taken into consideration. The results were very similar in water and in 8 M urea solutions.

TABLE IV
Number of Atoms, Groups, or Residues per Molecule

	Lysozyme	Conalbumin	Ovomucoid	Avidin
Mol. wt. assumed.....	14,900	87,000	28,800	66,000
Alanine.....	9.7	43	7.4	
Arginine.....	10.9	38	6.12	24.6
Aspartic acid.....	20.4	87	28.1	48
Cystine.....	(4.96)*	13.8	8.0	1.26
Glutamic acid.....	4.36	70	12.7	29.6
Glycine.....	11.3	66	14.6	40.4
Histidine.....	1.00	14.4	3.99	4.08
Isoleucine.....	5.91	33	3.14	27.7
Leucine.....	7.84	58	11.2	24.7
Lysine.....	5.81	60	11.8	28.0
Methionine.....	2.06	11.8	1.84	6.24
Phenylalanine.....	2.82	30	5.07	23.6
Proline.....	1.81	37	6.80	9.40
Serine.....	9.51	52	11.5	28.3
Threonine.....	6.89	43	13.3	58.2
Tryptophan.....	7.73	12.8	≤0.4	17.4
Tyrosine.....	2.94	22	5.06	3.21
Valine.....	6.11	61	14.8	23.7
Amino N.....	7.88	69	14.0	52
Amide N.....	18.1	65	20.6	61
Total N.....	198	1032	270	697
N accounted for by amino acids and amide†.....	195	1047	233	588
Total S.....	11.8	50	20	20
S accounted for by cystine and methionine†.....	12	40	18	8
Mol. wt. by summation of con- stituents†, ‡.....	14,455	85,186	18,851§	47,183§
Acid groups.....	(10)	123	39	53
Basic ".....	19.4	122		57
Peptide chains				
By free amino groups†,	2	9	2	<24¶
" basic groups†, **	1	10		0 (17)**
" acid " †, ††	(1)	9	14	33 (16)††

* Fromageot and Privat de Garilhe's value (23) of 8.0 per cent cystine is used in the calculations.

† All experimental values have been rounded off to the nearest integers before summation.

TABLE IV—*Concluded*

‡ The amino acids, amide ammonia, and phosphoric acid (avidin only) were totaled, 1 molecule of water was deducted for each group (2 for each cystine), and 1 molecule of water was added for each peptide chain (as calculated from the free amino groups).

§ Ovomuroid and avidin contain carbohydrate.

|| The number of peptide chains is assumed equal to the number of free amino groups less the number of lysine residues.

¶ The number of peptide chains would be less than twenty-four by the number of free amino groups in the nucleic acid component.

** The number of peptide chains is assumed equal to the number of basic groups less the number of arginine, histidine, and lysine residues. The value given in parentheses for avidin is calculated by adding the seventeen phosphate groups to the experimentally determined number of basic groups.

†† The number of peptide chains is assumed equal to the number of acid groups plus the number of amide groups less the number of aspartic acid, glutamic acid, and tyrosine residues. The value given in parentheses for avidin is obtained by deducting the seventeen phosphate groups from the experimentally determined number of acid groups.

but for only 66 per cent of the weight suggests that the non-protein moiety actually represents more than 30 per cent rather than 20 to 25 per cent (2) and thus may contain unknown constituents, possibly including free acid groups. This would explain both the disagreement between terminal amino and carboxyl groups and the low isoelectric point of ovomucoid (pH 3.8 (32)), which appears out of line with its proportions of acid and basic amino acids (25:22).

The data given in Table II for *avidin* are provisional because the material is not completely homogeneous by electrophoresis and because the small amount of material available did not permit extensive replication of assays. Nevertheless, the least frequent amino acid residues approach integral values (Table IV) on the basis of the tentative molecular weight of 66,000 found by osmotic pressure measurements.⁸ Much remains to be determined with respect to the composition of avidin, since only 85 per cent of the nitrogen, 72 per cent of the solids, and 40 per cent of the sulfur have been accounted for. Lysine accounts for only 28 of the 52 equivalents of free amino groups per mole. The high isoelectric point of avidin (around pH 10) appears inconsistent with the small excess of basic groups over acid groups, as determined by the dye technique. The recent finding⁹ that avidin contains nucleic acid may help to resolve these difficulties.

Phosvitin and *vitellin* (4, 3) are not pure proteins, and so a detailed discussion will not be offered. Mecham and Olcott (4) believe that the phosphorus content of vitellin is due in part to the presence of phosvitin.

⁹ H. Fraenkel-Conrat, in preparation for publication.

Part of the relatively high serine content found for vitellin accordingly would have been contributed by the serine-rich phosvitin.

SUMMARY

Amino acid and other analyses are presented for the following egg proteins: crystalline lysozyme, electrophoretically homogeneous conalbumin and ovomucoid, and the less pure proteins, avidin, phosvitin, and vitellin. Analyses are also presented for the following crystalline reference proteins: β -lactoglobulin, chymotrypsinogen, bovine serum albumin, and egg albumin; and the microbiological assay methods used are described.

We wish to acknowledge the technical assistance of Mrs. P. A. Thompson and Mrs. F. C. Marsh with the microbiological assays, and Mr. E. D. Ducay and Mrs. R. B. Silva with the chemical determinations. We are indebted to Mr. L. M. White for elementary analyses. We wish to thank Dr. M. S. Dunn of the University of California at Los Angeles for a gift of DL-isoleucine, and Dr. J. R. Spies of the Allergen Research Division of this Bureau for a gift of D- and L-methionine.

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THE SEPARATION AND ESTIMATION OF RIBONUCLEOTIDES IN MINUTE QUANTITIES*

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Studies on the composition and structure of nucleic acids in which this laboratory has been engaged for some time made it desirable to develop methods for the separation and quantitative determination of the mononucleotides, especially those formed from pentose nucleic acids by chemical or enzymatic hydrolysis. The literature is not rich in procedures that would lend themselves to a complete survey of the nucleotide constituents of nucleic acids.

The most promising approach appeared to be the employment of a chromatographic method, which was indicated by the successful application of filter paper chromatography to the separation and estimation of purines and pyrimidines (3). It might be mentioned that for separations on a preparative scale the use of exchange resins has proved of value for the characterization of ribonucleotides (4-6), as have starch columns for nucleoside separation (7).

The ribose mononucleotides failed to migrate in the solvent mixtures, such as aqueous *n*-butanol with and without the admixture of diethylene glycol, used for the separation of purines and pyrimidines (3) and also of the nucleosides.¹ Their chromatographic separation could, however, be effected in a solvent system consisting of aqueous isobutyric acid buffered with ammonium isobutyrate. In this solvent mixture muscle adenylic acid, yeast adenylic acid, and cytidylic acid could be separated from each other and also from an adsorption zone shared on the chromatograms by guanylic and uridylic acids.

In the preliminary experiments reported previously (1) the positions of the separated nucleotides on the chromatogram were made visible by an indirect method; namely, by the conversion of their uranium salts to brown

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¹ Unpublished experiments.

uranyl ferrocyanide. A brief description of this procedure is included here, since it may be of general interest for the demonstration of compounds which form insoluble uranium salts. For the detection of purines and pyrimidines and their derivatives (nucleosides, nucleotides, etc.), however, a much simpler direct method is now available. This procedure, which

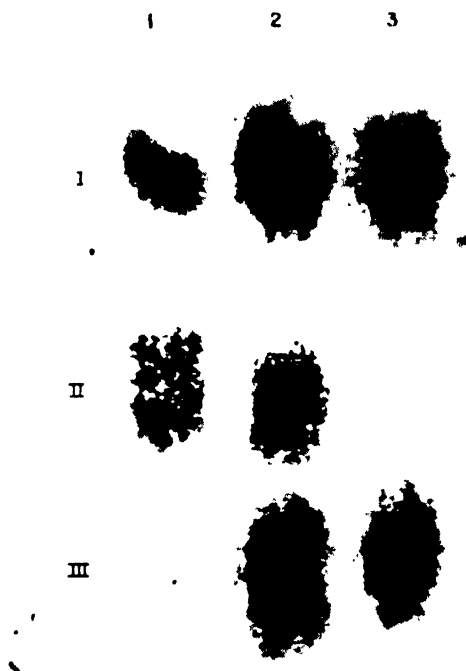


FIG. 1. Chromatogram observed in the ultraviolet light of the Mineralight lamp. Lane 1 shows the separation of uridylic acid (Section I) and cytidylic acid (Section II). In Lane 2 an alkaline hydrolysate of yeast ribonucleic acid is separated into guanylic and uridylic acids (Section I), cytidylic acid (Section II), and adenylic acid (Section III). Lane 3 presents the separation of guanylic acid (Section I) and yeast adenylic acid (Section III). Each zone corresponds to about 50 γ of nucleotide, with the exception of Section I of Lane 2 which contains the double quantity.

was applied in all experiments described here, also obviates, except under special circumstances, the demonstration of the separated free purines and pyrimidines via their mercury salts (3) and does away with the necessity of using guide strips in the chromatographic estimation of any of the nitrogenous nucleic acid constituents. A lamp emitting short wave ultra-

violet light, which is commercially available, served for the detection of the separated nucleotides on the chromatogram.² The purines and pyrimidines and their derivatives appear as dark absorption shadows on the background of the faintly fluorescing filter paper (Fig. 1). The amount of nucleotide detectable by this method varies from about 2 γ of adenylic acid to about 5 γ of cytidylic acid and depends, of course, to some extent upon the compactness of the adsorbate.

When test mixtures of the four ribonucleotides or alkaline hydrolysates of pentose nucleic acids (9) are subjected to separation, the adsorbates are found to occupy the following relative positions on the chromatogram. If the distance of the yeast adenylic acid from the starting point is taken as 100, muscle adenylic acid is 75, cytidylic acid 60, and guanylic plus uridylic acid 40, with aqueous isobutyric acid in an NH_3 atmosphere as the solvent mixture. These values are subject to considerable fluctuations, especially as regards cytidylic acid, and should be accepted as mere approximations. With a somewhat modified solvent system, likewise described below, in which by the addition of a definite amount of ammonia to the aqueous isobutyric acid a pH of 3.6 to 3.7 is established previous to chromatography, more constant mobilities are observed; *viz.*, yeast adenylic acid 100, muscle adenylic acid 87, cytidylic acid 75, and guanylic plus uridylic acids 50. The recently described isomer of adenosine-3-phosphoric acid (4, 6) went to the same position as did yeast adenylic acid.³

The separated nucleotides were eluted with m phosphate buffer of pH 7.1; the ultraviolet spectra of the extracts were determined and the nucleotide concentrations computed from the extinction values by procedures similar to those previously described for the purines and pyrimidines (3). Guanylic and uridylic acids were extracted together, but their respective concentrations could be estimated from the extinction values of the mixed eluate at two different wave-lengths by means of simultaneous equations based on the absorption of the pure nucleotides (Fig. 2). Data on nucleotide recovery and other details will be found in the experimental part. In general it can be said that when mixtures of all four nucleotides are separated with aqueous isobutyric acid in an NH_3 atmosphere a practically complete recovery of the pyrimidine nucleotides is observed, whereas the purine nucleotides are recovered to the extent of about 91 per cent. In the ammonium isobutyrate-isobutyric acid system buffered at pH 3.6 the recovery of guanylic acid, too, is almost complete. The application of the

² We are greatly indebted to Dr. C. E. Carter, Oak Ridge National Laboratory, who drew our attention to this instrument (Mineralight, Ultraviolet Products Corporation, Los Angeles, California). A similar arrangement was recently described by Holiday and Johnson (8).

³ We are very grateful to Dr. C. E. Carter for a specimen of this substance.

method to the investigation of the nucleotide composition of several pentose nucleic acids is described in the following paper (9).

The understanding of the conditions governing partition chromatography on filter paper (10), difficult enough in the case of a mixture of polar compounds (11), meets particular obstacles when several polyvalent ampholytes, such as the nucleotides, are to be separated. The sequence in which, in the course of a separation of a mixture of amphoteric substances, the individual components align themselves on a paper chromatogram will in the main be determined by two factors: (1) the type of ionic species in

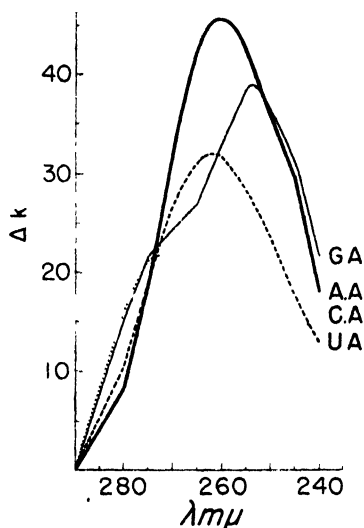


FIG. 2. Absorption spectra of the ribonucleotides in *m* phosphate buffer of pH 7.1. Δk is the difference between the specific extinctions at the particular wavelength and at 290 *mμ*. *G. A.*, guanylic acid; *A. A.*, adenylic acid; *C. A.*, cytidylic acid; *U. A.*, uridylic acid.

which the particular ampholyte occurs at the respective hydrogen ion concentrations prevailing in the stationary and mobile phases of the solvent system; and (2) the partition coefficient governing the distribution of the particular ionic species of the compound between the two phases as they exist on the paper. A third factor may under certain circumstances have to be added; namely, the contribution made by the paper itself acting as an adsorbent. As regards the ribonucleotides, only the first of these conditions can be partly appraised, thanks mainly to the work of Levene and Simms (12) on the dissociation constants of these compounds.

The mixture to be separated is dispensed at pH 5 when the nucleotides occur mainly as the monobasic salts. The separation is carried out in aqueous isobutyric acid buffered to pH 3.6 to 3.7 with ammonium iso-

butyrate. It can be shown that under these conditions pH values lower than those of the original solvent system prevail on the paper. At a pH somewhat below 3 the amino group of cytidylic acid will be dissociated almost completely and that of adenylic acid to a large extent; *i.e.*, these nucleotides, being near their isoelectric points, will exist principally as dipolar ions. Guanylic and uridylic acids, on the other hand, will distribute themselves as the monoammonium salts.

The reasons for the particular conditions chosen for the separation procedures discussed here will be made clearer by the inspection of Fig. 3. The R_F values (10) of the nucleotides, *i.e.* the proportion of the distances of the starting point from the adsorbate and from the solvent front, are

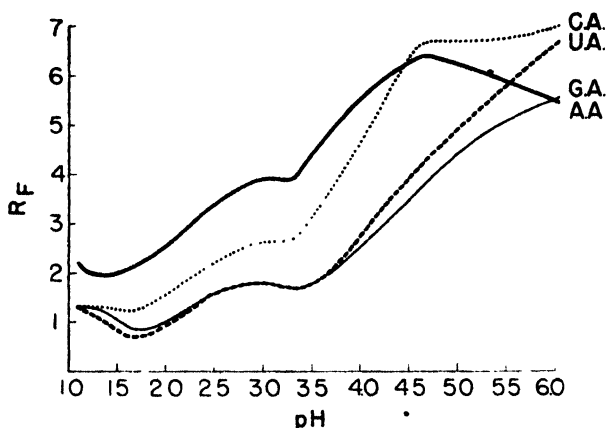


FIG. 3. R_F values of the ribonucleotides as a function of the pH of the solvent system. Symbols as in Fig. 2.

illustrated as a function of the pH of the solvent mixture. All four nucleotides gave curves that were, in general appearance, quite similar. These curves show that the pH of 3.7, chosen for the solvent mixture used in the quantitative separations, is most favorable with respect to the positions of the nucleotides. It will be noticed that cytidylic acid is in its chromatographic mobility more sensitive to a rise in pH in this range than are the other nucleotides and moves rather rapidly into the adenylic acid zone with increasing pH (Fig. 3). A solvent mixture more acidic than pH 3.7 would, on the other hand, hardly have been practicable, not only because of the retarded migration of all nucleotides at greater acidity, but also because of the danger of partial cleavage of the purine nucleotides under these conditions.

As our knowledge of the chromatographic behavior of ampholytes of the type of the nucleotides increases, better solvent systems for their separation

will doubtless be found. The principal limitation of the present method may be seen in the fact that guanylic and uridylic acids are found together.⁴ The indirect spectroscopic procedure, employed in this study for the individual estimation of these two nucleotides, gives, however, adequate results, unless one nucleotide is present in a very large excess. In the latter case the pyrimidine nucleotides can be separated and determined quantitatively following the hydrolysis of the purine nucleotides, as will be shown in the following paper (9).

EXPERIMENTAL

Materials

Ribose Nucleotides—The preparation from yeast and the characterization of the *adenylic acid*, *guanylic acid* (basic sodium salt), and *cytidylic acid* employed have been described previously (13). The *muscle adenylic acid* (adenosine-5-phosphoric acid) used was a commercial preparation (Ernst Biscoff Company, Inc., Ivoryton, Connecticut).

Uridylic acid was prepared by the deamination of cytidylic acid according to Brederick (14) and purified by conversion to the brucine salt which was decomposed with ammonium hydroxide. The resulting *diammonium uridylate*, after recrystallization from aqueous methanol, melted (with decomposition) at 183–185° (uncorrected) and had a rotation of $[\alpha]_D^{22} = +22.3^\circ$ (0.35 per cent solution in water). Levene (15) reported for this compound a melting point of 185° and $[\alpha]_D^{20} = +21.0^\circ$.

$C_9H_{13}N_4O_8P \cdot \frac{1}{2}H_2O$ (367.3)

Calculated. N	15.2, Ammonia N 7.6, P	8.4
Found. " (Dumas)	14.8, " " 7.1, " (gravimetric)	8.5

The properties of the nucleotides employed in the present experiments are summarized in Table I. The content in free nucleotide of the various preparations, required for the computation of the optical characteristics, was calculated from the analytically determined nitrogen values. The ultraviolet absorption spectra of the nucleotides, dissolved in *m* phosphate buffer of pH 7.1, were measured by means of a Beckman photoelectric quartz spectrophotometer. For the construction of the absorption spectra,

⁴ Preliminary experiments by one of us (D. E.) have demonstrated the existence of a number of solvent systems in which the chromatographic separation of the now faster moving uridylic acid from guanylic acid can be performed. Such solvent mixtures consist, for instance, of NH_3 -containing aqueous alcohols (ethanol, *n*-propanol, *n*-butanol) (compare (11)) or of alcohols containing acetic acid buffered with ammonium acetate. Following the separation by the method described in this paper, the joint guanylic-uridylic acid adsorbate is irrigated with one of the solvent systems mentioned, most conveniently by means of a transfer technique in which the paper rectangle containing the joint adsorption zone is cut out and fastened suitably to the top of a fresh paper strip.

reproduced in Fig. 2, the extinction values are expressed as Δk , the difference between the specific extinction at the particular wave-length and the specific extinction at 290 $m\mu$. The reasons for the use of this expression have been discussed previously (3); its application will be exemplified later. The specific extinction is defined as $k = E/cl$, where E is the extinction, c the concentration of the solute in gm. per liter, and l the thickness of the absorbing layer in cm. The optical characteristics of the pyrimidine nucleotides shown here are in good agreement with recently published ultraviolet absorption spectra of these compounds (16) that came to our attention after the present work was completed.

TABLE I
Ribose Nucleotides

Compound	N	P	Atomic N:P ratio	Content in free nucleotide (based on N content)	Absorption maximum	Δk_{\max}^*
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>mμ</i>	
Basic sodium guanylate.....	13.27	6.16	4.8	68.8	254	38.2
Adenylic acid.....	18.54	8.45	4.9	91.9	259	45.3
Cytidylic acid.....	12.40	9.54	2.9	95.4	270	21.2
Diammonium uridylate.	7.70†	8.52	2.0	89.1	262	31.3

* Δk_{\max} is the difference between the *specific* extinctions at the absorption maximum and at 290 $m\mu$ (compare (3)). The values reported are for the free nucleotides in M phosphate buffer of pH 7.1.

† Pyrimidine nitrogen (total N minus ammonia N).

Solvent and Filter Paper—Commercial *isobutyric acid* (Pafagon Division, The Matheson Company, Inc., East Rutherford, New Jersey), when necessary rectified by distillation, was employed. The *filter paper* used for chromatography was Schleicher and Schüll, No. 597.

Separation and Quantitative Estimation of Ribose Nucleotides

Solutions—The nucleotides were employed as aqueous solutions, as a rule adjusted to pH 5. Their concentration was 0.1 to 0.3 per cent with respect to each free nucleotide.

Separation—The method of separation followed closely the procedures described previously for purines and pyrimidines (3). Paper sheets, 15 cm. wide and 50 cm. long, carried four longitudinal lanes 3.7 cm. wide and ended, if the solvent was to be permitted to overrun the lower border, in a series of teeth cut into the latter. A line drawn across the sheet 8 cm. below its upper edge marked the starting points at which accurately measured quantities of solution were deposited in the centers of three of the

lanes by means of a Gilmont ultramicro burette (Emil Greiner Company, New York). The fourth lane was left free. Normally, 0.01 cc. of solution was used. For larger volumes, up to 0.04 cc., it was found desirable to apply the solution in 0.01 cc. lots which were allowed to dry on the paper between additions.⁵

The separation experiments were carried out at room temperature with the same arrangement of jar and trough as described before (3).

Solvent Systems—Two solvent mixtures were employed in the course of this work. The first system, designated here as the "acid system," required a suitably maintained NH_3 atmosphere in the jar during separation. It consisted of isobutyric acid saturated with water at 15°; this corresponds to a mixture of 11 volumes of isobutyric acid and 6 volumes of water. A small beaker containing about 3 cc. of water saturated with isobutyric acid, obtained as the heavier layer in the preparation of the solvent, was centered in the bottom of the jar. Two 30 cc. beakers, each containing 10 to 15 cc. of N ammonia, also were placed at the bottom on opposite sides. The separation was allowed to proceed for about 18 hours, at which time the solvent front usually had passed the lower edge of the paper.

In the second solvent system used, to which reference will be made as the "buffered system," the rate of movement of the separated nucleotides was less dependent upon the temperature of the experiment. This solvent mixture, which in general appears preferable, consisted of 10 volumes of isobutyric acid brought to pH 3.6 to 3.7 by the addition of 6 volumes of 0.5 N ammonia. A beaker containing the same solvent was placed at the bottom of the jar. The period of irrigation was 12 to 15 hours, the temperature 21–25°.

The relative positions on the chromatogram of the separated nucleotides were mentioned in the beginning of this paper and are discussed in greater detail at the end.

Location of Adsorbates—The paper sheets were first dried in air for about $\frac{1}{2}$ hour and then in an oven at 85° for 20 minutes. The positions of the nucleotides on the chromatogram were determined by holding the paper over an ultraviolet lamp² in a dark room. The individual adsorption zones, which stood out as dark spots on the background of the faintly fluorescing paper, were then lightly marked with pencil. The lower limit of detectable quantities ranges from 2 γ of adenylic acid to 5 γ of cytidylic acid. The appearance of a typical chromatogram in the ultraviolet light of the Mineralight lamp is shown in Fig. 1.⁶

⁵ In many drying operations on paper an electric hair dryer was found useful.

⁶ We are greatly indebted to Mr. L. W. Koster of the Department of Neurology for the preparation of the photograph. A contact print taken in the transmitted light of the Mineralight lamp served as the negative. An ultraviolet photograph of a chromatogram of nucleic acid derivatives has been published recently (17).

The nucleotides could also be demonstrated by a chemical method of development. The paper strips were pulled slowly through a 0.15 per cent solution of uranyl acetate in water and then washed in a beaker with running water for 20 seconds. In this manner the nucleotides were fixed on the paper as the uranyl salts. The strips were then passed slowly through a 3 per cent aqueous solution of potassium ferrocyanide, acidified immediately before the experiment with N HCl. This resulted in the appearance of red-brown spots of uranyl ferrocyanide (18), indicating the position of the separated nucleotides. Amounts of the order of 20 γ of nucleotide thus could be demonstrated. Neither purines and pyrimidines nor nucleosides gave this reaction.

Extraction—The four lanes on each paper were cut apart and rectangles 4 to 6 cm. long, with the predetermined nucleotide zones as the centers, were removed. Rectangles of the same size and at corresponding positions were cut from the fourth lane that had been left free; their extracts served as the blanks in the spectrophotometric determination of the respective nucleotides. Each paper segment was extracted in a small stoppered test-tube (13 \times 100 mm.) with exactly 4 cc. of M phosphate buffer of pH 7.1 at 37° for a period of about 18 hours. The extracts, well mixed and cooled to room temperature, were centrifuged immediately before spectroscopy.

Ultraviolet Spectroscopy—The absorption measurements were carried out as described before (3). The extinction values were determined at 290 $m\mu$, at the wave-length of maximum absorption of each nucleotide (see Table I) and at 5 $m\mu$ above and below the latter, in order to verify the position of the maximum. When contamination of an extract by other nucleotides was suspected, its entire ultraviolet spectrum was determined and compared with the spectra of the pure nucleotides (Fig. 2). The nucleotide contents of the extracts were calculated by the following expression.

$$\text{Nucleotide (in micrograms per 4.0 cc. of extract)} = 4000(E_{\max.} - E_{290})/\Delta k_{\max.}$$

$E_{\max.}$ and E_{290} are the extinction values ($\log I_0/I$), as found for the particular extract at the point of maximum absorption and at 290 $m\mu$ respectively; $\Delta k_{\max.}$ is given in Table I for each nucleotide, as are the positions of the absorption maxima.

When alkaline hydrolysates of pentose nucleic acids or test mixtures of the four corresponding nucleotides were subjected to separation, guanylic and uridylic acids occupied the same position on the chromatogram, as has already been emphasized above, and were, therefore, extracted together. The respective concentrations of the two components in the extract can, however, be determined by means of the specific extinction of the pure nucleotides at 245 and 265 $m\mu$ (compare Fig. 2) and of the extinction of the

II) the same relative recoveries were recorded. It will be seen that in this solvent mixture the pyrimidine nucleotides are recovered practically quantitatively, and the purine nucleotides to the extent of 91 per cent. The spread of the individual figures was small. The results of experiments with the "buffered system" are reproduced in Tables III and IV. The recoveries observed when single nucleotides are subjected to chromatography are assembled in Table III, whereas several separation experiments are compared in Table IV. This solvent mixture, buffered to pH 3.6 to 3.7, permits the practically quantitative recovery of guanylic, uridylic, and cytidylic acids, the latter giving values that are slightly too high; adenylic acid, however, suffers a loss of about 9 per cent in both solvent

TABLE III
Quantitative Chromatography of Single Ribose Nucleotides in "Buffered System"

No. of experiments	Subjected to chromatography		Recovered after chromatography (as average per cent of initial weight)*
	Nucleotide	Quantity	
3	Guanylic acid	9.4	92
9	" "	15.9	98
3	Adenylic acid	13.2	88
12	" "	27.5	93
3	Cytidylic "	15.7	96
3	" "	19.1	102
3	" "	66.8	99
3	Uridylic "	11.3	99
6	" "	22.6	99

* The average per cent of nucleotide recovered in all experiments, with its standard deviation in parentheses, guanylic acid, 97 (4); adenylic acid, 92 (2); cytidylic acid, 99 (3); uridylic acid, 99 (1).

systems. Somewhat elevated figures for cytidylic acid were observed only when nucleotide mixtures were separated (Tables II and IV); this may have resulted from a small amount of contamination by other nucleotides. The loss in adenylic acid cannot yet be fully explained. This nucleotide is the component moving most rapidly on the chromatogram and it may be that traces of it are retained by the paper on its path; or, but this is less likely in view of the very small spread of the analytical results in Tables II to IV, a small portion of the adenylic acid may have been degraded by acid hydrolysis in the course of the chromatographic separation.

pH Dependence of Chromatographic Nucleotide Distribution

pH of Effluent Solvent—A few orienting experiments were carried out to indicate the effect that the distribution of the buffered solvent system on

the filter paper surface had on the composition of the mobile phase. The solvent mixture employed in all cases consisted of 10 volumes of isobutyric acid and 6 volumes of 0.5 N ammonium hydroxide and had a pH of 3.67. When a paper sheet (15 × 50 cm.) with the lower end tapering to a collection point was irrigated with an excess of solvent in the trough in the usual arrangement and the overflow collected from the bottom of the strip, the liquid that had overrun within 16 hours after the start of the experiment (1.6 cc.) had a pH of 3.34; after another 10 hours, 2 cc. with a pH of 3.53 were collected; the excess solvent remaining in the trough at this time (8.8 cc.) showed a pH of 3.82. In another run, with a smaller amount of solvent fed to the paper, so that none remained in the trough, 2.4 cc. of overflow were collected with a pH of 3.77.

TABLE IV
Separation of Ribose Nucleotides in "Buffered System"

Six experiments each.

Nucleotide in mixture subjected to separation				Nucleotide recovered after separation (as average per cent of initial weight)*			
Guanylic acid	Adenylic acid	Cytidylic acid	Uridylic acid	Guanylic acid	Adenylic acid	Cytidylic acid	Uridylic acid
γ	γ	γ	γ				
8.9	12.6	14.3	11.1	101	91	107	95
15.9	27.5	19.1	22.6	103	90	104	93

* The average per cent of nucleotide recovered in all experiments, with its standard deviation in parentheses, guanylic acid, 102 (5); adenylic acid, 90 (1); cytidylic acid, 106 (3); uridylic acid, 94 (3).

These and similar experiments not reported here gave indications that the composition of the phases, as they formed in the course of a chromatographic separation experiment, was discontinuous, and that the stationary phase probably had a pH lower than that of the initial solvent mixture, whereas the composition of the mobile phase varied in the course of the experiment from a pH lower than that of the initial solvent at the beginning of the run to one as high or even higher at the end.

Relative Positions of Nucleotides on Chromatogram in Solvents of Different Acidity—Although the determination of R_F values (10), i.e. the proportion of the distances of the starting point from the adsorbate and from the solvent front, yields, in the case of the nucleotides, results that are even less constant than those from other classes of compounds, it appeared of interest to study the effect of solvent systems of different pH values on the relative migration rates of the several ribonucleotides. The temperature of the experiments was, by a crude thermostatic arrangement, maintained

at 25–28°. Eighteen different solvent mixtures were employed, covering a pH range from 1.08 to 6.12. Between pH 2.44 and 6.12, mixtures of 10 volumes of isobutyric acid and 6 volumes of water, containing varying quantities of NH_3 , were used; at lower pH values the proportions of isobutyric acid and water (containing NH_3 or HCl) were 11:6 and 11.5:6. The solvent mixture employed at pH 1.63 consisted only of 11 volumes of isobutyric acid and 6 volumes of water. Individual solutions of adenylic, guanylic, cytidylic, and uridylic acids were chromatographed side by side on the four lanes of each paper sheet. Each jar contained two sheets. Two R_F values were thus determined for each nucleotide which had been so placed as to occupy an outer lane on one sheet, an inner lane on the other. The figures reported here represent the averages. At the beginning of each run 12 cc. of solvent were put into the trough and two beakers, each containing 3 cc. of the solvent, were placed at the bottom of the jar. After the entire solvent had passed from the trough into the paper sheets, the papers were removed, the positions of the solvent boundaries were marked, and the adsorption zones outlined on the dried sheets under the ultraviolet lamp.

The R_F values of the nucleotides, observed as a function of pH, are shown graphically in Fig. 3. It should be mentioned that the characteristic shapes of these curves were more exactly reproducible than the individual numerical values on which they are based. Muscle adenylic acid yielded curves that were very similar in shape to those of yeast adenylic acid; its R_F values, however, were lower, averaging about 85 per cent of the figures for yeast adenylic acid.

SUMMARY

The chromatographic separation on filter paper of the ribonucleotides with aqueous isobutyric acid buffered with ammonium isobutyrate as the solvent and their quantitative estimation are described. Yeast adenylic acid can be separated from muscle adenylic acid and also from guanylic, cytidylic, and uridylic acids. Although, when all four nucleotide constituents of yeast ribonucleic acid are to be separated, guanylic and uridylic acids are found to share the same position on the chromatogram, procedures permitting the complete nucleotide analysis of a pentose nucleic acid have been developed. The separation and estimation of quantities ranging from 8 to 70 γ of nucleotide are described; the recovery is practically complete, with the exception of adenylic acid which is recovered to the extent of 92 per cent. Methods for the demonstration on the chromatogram of the separated nucleotides are discussed. Some of the problems underlying the separation of nucleotides by filter paper chromatography are considered and experiments are presented to illustrate the dependence upon pH of the distribution of these substances on the chromatogram.

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NUCLEOTIDE COMPOSITION OF PENTOSE NUCLEIC ACIDS FROM YEAST AND MAMMALIAN TISSUES*

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The problems presented by the pentose nucleic acids of mammalian cells, and even by the ribose nucleic acid of yeast, are far from solved. Whereas the desoxypentose nucleic acids, isolated under as mild conditions as can be devised, can now be regarded as high polymers of a composition characteristic for the species from which they are derived (2), no comparable preparations of pentose nucleic acids, with the possible exception of those from plant viruses (3, 4), are available. Whether what is isolated from a cell as pentose nucleic acid represents one definite compound or a degradation product of such a compound, originally present in a more highly polymerized form, whether the preparations available at present, consisting of nucleotide aggregates of widely differing molecular sizes, are identically composed fragments of the same mother substance or representatives of locally and functionally different components of the cell, or to what extent pentose nucleic acids vary not only in quantity, but also in composition with the developmental stage of the cell, all this cannot be decided before better methods of isolation, characterization, and analysis are available.

The micromethods for the study of the purine and pyrimidine composition of nucleic acids, developed in this laboratory (5), have already been applied to a number of desoxypentose nucleic acids from a variety of cellular sources (2, 6-8) and to pentose nucleic acids from yeast and pig pancreas (9). It appeared desirable to resume the study of the composition of pentose nucleic acids by means of a method permitting the estimation of their component ribonucleotides and to extend it to preparations of pentose nucleic acid from the livers of different species.

The analytical methods employed here have been described in the pre-

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ceding paper (10). The procedures used for the hydrolysis of the pentose nucleic acids are in essence based on old observations demonstrating the readiness with which these nucleic acids are cleaved to mononucleotides by weak alkali (11-13).

EXPERIMENTAL

Materials

Ribose Nucleic Acid of Yeast—Four different preparations of yeast ribonucleic acid were used. *Preparation 1* was a purified commercial sample (Merck); its purification has already been described, since it served for the purine and pyrimidine determinations reported previously (9). *Preparation 2* was prepared from another commercial specimen (Schwarz Laboratories, Inc., New York) by procedures similar to those used for Preparation 1, with an additional purification step: the solution of the ammonium nucleate was dialyzed against running tap water, the nucleic acid precipitated with acidified alcohol (9), and its aqueous suspension again dialyzed against running tap water and ice-cold distilled water. *Preparation 3* was isolated from fresh bakers' yeast by procedures modeled exactly after those described below for the preparation of pentose nucleic acids from animal livers. 1 kilo of yeast cake yielded 1.7 gm. of this nucleic acid preparation.

Preparation 4 was made from freshly ground, defatted bakers' yeast. The yeast cells (95 gm.) were washed with 0.14 M sodium chloride, and then with 50, 75, 95, and 100 per cent ethanol. Their suspension in equal volumes of 0.14 M NaCl and absolute alcohol was passed through an ice-cooled wet crushing mill for bacteria (14) and 2 volumes (240 cc.) of 70 per cent ethanol were added to the mixture. The precipitate of mostly crushed cells was washed repeatedly with 80 and 95 per cent ethanol, ethanol-ether (1:1), and ether, and dried *in vacuo*. It was twice extracted with 100 cc. portions of 10 per cent aqueous sodium chloride at 90° for $\frac{1}{2}$ hour, and 2 volumes of ethanol were added to the combined centrifuged extracts. The resulting precipitate, washed with dilute and absolute alcohol and ether and dried, weighed 0.71 gm. It was taken up in 35 cc. of water, the mixture was centrifuged, and 0.25 volume of 20 per cent barium acetate solution (pH 7) and 1 volume of ethanol were added to the supernatant. The precipitate resulting from the centrifugation of the chilled mixture was washed with 5 per cent barium acetate and its aqueous suspension (17 cc.) stirred in a high speed mixer in the presence of a small excess (150 mg.) of sodium sulfate. The solution, clarified by centrifugation, was freed of protein by being stirred six times in a high speed mixer with chloroform-octanol (9:1) and then was poured into 2 $\frac{1}{2}$ volumes of ice-cold ethanol (50 cc.) that was made 0.05 N with respect to HCl. The

mixture was chilled overnight and the precipitate, after being washed with alcohol, was suspended in 20 cc. of water and brought into solution by the cautious addition of dilute ammonia to pH 6. The precipitation with acidified alcohol was repeated and the nucleic acid washed with 80 and 100 per cent alcohol and ether and dried, when 0.17 gm. of an almost white powder was obtained.

Some of the properties of these nucleic acid preparations are summarized in Table I.

Pentose Nucleic Acid from Pig Pancreas—The preparation of this substance, together with the analytical results on its constituent purines and

TABLE I
Pentose Nucleic Acids

Preparation No.	Source	N	P	Pentose nucleic acid	Desoxy-pentose nucleic acid	Ultraviolet absorption maximum	
		per cent	per cent	per cent	per cent	m μ	$\epsilon(P)$
1	Yeast	15.3	8.0			257.5	9,800
2	"	15.5	8.6			258	9,800
3	"	15.0	8.6			258	10,000
4	"	14.3	8.5		2	258	8,500
5	Pig pancreas	15.4	7.9		3	256	9,800
6	" liver	15.8	8.7	91	8	258	8,700
7	Sheep liver	15.1	8.4	86	9	257.5	9,100
8	Calf "	14.2	7.7	91	3	257	9,900
9	Beef "	14.6	7.8	81	7	258	10,100
	Carcinomatous human liver						
10	Unaffected tissue	14.9	8.5	73	16		
11	Metastases	13.2	6.8	47	30		

pyrimidines, has already been reported (9). This nucleic acid specimen is listed here as Preparation 5.

Pentose Nucleic Acids from Liver of Pig, Sheep, and Ox—The procedures used for the isolation of these fractions represent a modification of the method employed originally by Clarke and Schryver (15) for the preparation of yeast ribonucleic acid and applied later by Davidson and Waymouth (16) to the pentose nucleic acid of sheep liver. The fresh tissues serving for the preparations weighed between 500 and 2000 gm. They were cut into small pieces and ground for 1 minute in a total of about 3 volumes of cold 95 per cent ethanol (*i.e.*, 1 liter of alcohol for 350 gm. of tissue) by means of a high speed mixer equipped with cutting blades. The mixture was kept at room temperature for 24 to 48 hours and the tissue powder extracted twice more, first with 2 volumes of ethanol and then with 2

volumes of ethanol-ether (1:1), each time for 24 to 48 hours. The powder was removed by suction, washed with ether, and dried in air. Its weight amounted to 25 per cent of the fresh tissue in the case of pig and sheep livers, to 34 per cent with beef liver, to 39 per cent with calf liver.

The dry liver powder was ground for 4 minutes with 4 volumes of 10 per cent aqueous sodium chloride in the high speed mixer and the mixture refrigerated for 24 hours. The sediments, obtained by centrifugation in the cold at $1900 \times g$ for 10 minutes, were twice more extracted in the mixer with 2 volumes and 1 volume respectively of 10 per cent NaCl. The second and third extracts often were more viscous than the first.¹ The combined extracts, clarified by centrifugation, were poured into twice their volume of cold 95 per cent alcohol. This produced an almost immediate, copious formation of slimy fibers, consisting principally of desoxypentose nucleoprotein (17, 18), that were lifted at once or after cooling by being spooled on a glass rod attached to the shaft of a very slowly rotating stirrer.² The remaining alcohol-water mixture was kept in the refrigerator for 24 hours, during which time a granular precipitate settled. It was collected by centrifugation, washed repeatedly with 70 and 100 per cent ethanol, and dried *in vacuo*. The weight of this crude fraction corresponded to 0.3 to 0.6 per cent of the fresh liver. It was distributed in a mixer in 20 parts of water, the mixture was clarified by centrifugation at $2700 \times g$, and the sediment extracted once more with 15 parts of water. To the combined extracts 0.25 volume of 20 per cent aqueous barium acetate (pH 7) was added and the mixture chilled overnight. The precipitated barium pentose nucleate³ was collected and washed two to four times with 5 per cent barium acetate solution. It was then distributed in water by means of a high speed mixer, solid sodium sulfate was added, and the mixture was stirred for 5 minutes at high speed. (Per gm. of the alcohol-insoluble fraction that had served for the preparation of the Ba salt, 12 cc. of water and 100 mg. of anhydrous sodium sulfate, dissolved in a little water, were used.) Following the addition of chloroform-octanol (9:1), the stirring was continued for another 3 minutes and the mixture

¹ Very viscous extracts were also obtained when, following Davidson and Waymouth (16), the second extraction with 10 per cent NaCl solution was carried out at 90° for 30 minutes. No appreciable improvement in the yield of pentose nucleic acid was observed under these conditions.

² In previous work on the isolation of liver pentose nucleic acid (16) no mention is made of the precipitation at this stage of fibers containing desoxypentose nucleic acid, nor is the manner stated in which the latter was removed from the final preparations of pentose nucleic acid.

³ If much desoxypentose nucleic acid remained at this stage, the precipitation of the barium pentose nucleate was incomplete and the supernatant solution very turbid. Sometimes fractions were encountered that yielded a barium precipitate only after the addition of 1 volume of ethanol.

centrifuged. Four to eight deproteinization treatments usually were necessary.

The clear yellowish supernatant was dropped slowly with stirring into 3 volumes of very cold ethanol (-10°) that had been made 0.05 N with respect to HCl, the mixture was chilled overnight, and the gelatinous precipitate recovered by centrifugation and washed with 80 per cent ethanol. Its suspension in a small quantity of water was brought to pH 6 by the slow addition with cooling of 2 N ammonia. The precipitation of the solution, clarified by centrifugation, with acidified alcohol was followed once more by conversion to the ammonium salt and precipitation. The final precipitate was washed with 80 and 100 per cent alcohol and with ether, dried *in vacuo*, and ground in an agate mortar to a fine, almost white powder. The yields of the *liver pentose nucleic acids* varied from 0.02 to 0.03 per cent of the fresh organs. The pentose nucleic acid from pig liver is designated Preparation 6, that from sheep liver Preparation 7, and the fractions obtained from calf and beef liver Preparations 8 and 9 respectively.

Pentose Nucleic Acids from Normal and Carcinomatous Liver Tissue of Man—In view of the difficulties attending the acquisition of normal fresh human tissue it was thought best, for the time being, to examine a pathological specimen and to compare the pentose nucleic acids isolated from an unaffected portion of carcinomatous liver and from metastatic tissue of the same organ. The specimen, obtained through the courtesy of the Department of Pathology of this University, consisted of a part of a human liver that was riddled with metastases from the sigmoid colon. Portions of unaffected, normally pigmented hepatic tissue (270 gm.) and of the non-pigmented metastatic nodules (438 gm.) were dissected and served for the preparations.⁴ Since in this case both desoxypentose nucleic acid (8) and pentose nucleic acid were to be isolated from the same specimen, a different procedure (compare (17, 19)) was adopted.

The tissue was ground in cold 0.05 M sodium citrate solution (pH 7.4) for 90 seconds in a high speed mixer, the mixture was centrifuged at $1900 \times g$, and the sediments were washed seven times with 0.14 M sodium chloride solution that had been made 0.01 M with respect to sodium citrate, until the extracts gave no more than a very weak reaction for pentose. The washed tissue residues served for the preparation of desoxypentose nucleic acid. The combined filtered saline extracts were brought to pH 4.2 with acetic acid, when flocculation occurred. The mixture was chilled and centrifuged, the sediments were washed with 0.5 M NaCl and with water and brought into partial solution by the cautious addition to their aqueous

⁴ We are greatly indebted to Dr. Mogens Faber of Copenhagen for the preparation of the tissue specimens.

suspension of 0.5 M sodium bicarbonate until pH 7.8 was reached. The mixture was clarified by centrifugation and the supernatant again adjusted to pH 4.2. The resulting precipitate was washed with alcohol and alcohol-ether (1:1) and dried. It was extracted three times with 10 per cent sodium chloride solution, once at 90° for $\frac{1}{2}$ hour and twice in the cold. The addition of 2 volumes of ethanol to the combined extracts produced a granular precipitate which was collected after refrigeration, washed with alcohol and ether, and dried. The remainder of the procedures (precipitation as the Ba salt, conversion to the sodium and ammonium salts, precipitation of the nucleic acid, etc.) closely followed the methods described before for the pentose nucleic acids from animal livers. Both pentose nucleic acid preparations formed almost white powders. The specimen from the normal liver portion, obtained in a yield of 0.08 per cent of the tissue, is designated Preparation 10, that from the metastases (yield 0.05 per cent) Preparation 11.

Elementary Composition and Physical Properties—Data on the elementary composition of the pentose nucleic acids are given in Table I. The samples were, for analysis, dried in a high vacuum at 60° for 3 hours, and were weighed with exclusion of moisture. N was determined by the Dumas method, and P by the gravimetric Pregl-Lieb procedure.⁵ The desoxy-pentose nucleic acid content was estimated colorimetrically by means of the diphenylamine reaction (20), with a well purified preparation of potassium desoxyribonucleate of calf thymus (6) as the standard. The pentose nucleic acid content was determined by the orcinol reaction (21), the color given by the yeast ribonucleic acid Preparation 1 (Table I) serving as the standard of comparison. It will be understood that because of the higher concentration of purine nucleotides than of pyrimidine nucleotides in the mammalian pentose nucleic acids, as shown before (9) and, in much greater detail, in the present paper, pentose determinations based on a comparison with yeast ribonucleic acid will have only a relative, but not an absolute, validity. When, in parallel estimations of the pentose nucleic acid contents of Preparations 10 and 11, phloroglucinol (22) was substituted for orcinol, very similar values were obtained; namely, 78 per cent in Preparation 10, 46 per cent in Preparation 11. These two substances from carcinomatous human liver were least satisfactory from the analytical point of view; they were much more contaminated with desoxy-pentose nucleic acid than were the other preparations discussed here, probably because the unavoidable delay between the removal of the organ and its processing had permitted depolymerization to set in.

The ultraviolet absorption spectra were determined in phosphate buffer of pH 7.1, with the exception of Preparations 1 and 5 which were measured

⁵ We are indebted to Miss R. Rother for the microanalyses.

in aqueous solutions at pH 6.5 and 6.3 respectively. The expression $\epsilon(P)$ has been defined previously (23).

Quantitative Determination of Purine Nucleotides, Pyrimidine Nucleotides, and Purines in Pentose Nucleic Acids

Hydrolysis—Three procedures, the last one identical with the method for the estimation of free purines and pyrimidines described before (9), were employed for the characterization of the pentose nucleic acids.

Procedure 1—Amounts varying from 6 to 20 mg. of the nucleic acid (dried previously in a high vacuum at 60° for 3 hours) were weighed into small test-tubes, suspended in a few drops of water, and brought into solution by the addition of 1 drop of 33 per cent sodium hydroxide. The cautious addition of droplets of 33 per cent NaOH was continued until a pH of 13 to 14 (indicator paper) was reached. The stoppered tubes which now contained a volume of 0.2 to 0.3 cc., were kept for a minimum of 12 hours, usually overnight, at 30°. The nucleic acid hydrolysates usually were clear and colorless; in some preparations a minute amount of brown flocules settled which were removed by centrifugation. The hydrolysates were brought carefully to pH 5 with the use of concentrated and of N hydrochloric acid and adjusted with water to an exact volume of 1 cc.

Usually both total phosphorus and inorganic phosphorus (24) were determined in aliquot portions of these solutions. The quantity of inorganic P liberated in the hydrolysis was found to be negligible in all cases.

Procedure 2—Accurately weighed samples (6 to 14 mg.) of dried nucleic acid were introduced into the hydrolysis vessel described previously ((19), Fig. 1) and suspended in 0.5 to 1.0 cc. of absolute methanol. Dry HCl gas was passed through the mixture, which was kept at 50°, for about 3 hours. The total mixture, including the purine hydrochlorides that had precipitated in the course of the treatment, was evaporated to dryness at 45° under a current of nitrogen, with several additions of fresh methanol to remove most of the acid. The residue was suspended in a few drops of water, and the hydrolysis with alkali at pH 13 to 14 and the rest of the operations were carried out as described under Procedure 1.

Procedure 3—The free purines and pyrimidines were determined by the method previously described (5, 9).

Chromatographic Separation—The nucleotides were separated and estimated, by the methods described in the preceding publication (10), in an isobutyric acid system as the solvent. Most separations were carried out in the "acid system." The few cases in which the "buffered system" was employed are indicated in the tables. When the hydrolysis had been carried out according to Procedure 1, the hydrolysates of all pentose nucleic acids examined here exhibited the three characteristic zones of adsorption

(10). The adenylic acid spot occupied the position characteristic of yeast adenylic acid in all cases. It could, however, have included, in addition to adenosine-3-phosphoric acid, the recently described adenylic acid isomer (25, 26) which, as was pointed out before (10), goes to the same spot in the solvent systems used here. The admixture of muscle adenylic acid to hydrolysates irrigated with the "acid system" led to the appearance of a fourth adsorption zone that coincided with the position of this nucleotide, when run alone in parallel. No qualitative differences in the composition of the hydrolysates of any of the pentose nucleic acids examined could be detected.

When the hydrolysates were prepared by Procedure 2, which leads to the cleavage of the purine nucleotides, four adsorption zones were encountered, corresponding in position (in descending order) to uridylic and cytidylic acids, guanine, and adenine. Control experiments with test mixtures of these four components verified these findings; adenine, the fastest component, was found almost at the bottom of the paper strips. The separated components were extracted in the usual manner, the pyrimidine nucleotides with *M* phosphate buffer of pH 7.1 (10), and guanine and adenine with *N* and 0.1 *N* hydrochloric acid respectively (5).

Presentation of Results—The nucleotide composition of the several pentose nucleic acids examined here is, for the sake of brevity, expressed in one way only; namely, as per cent of the organic phosphorus of the preparation accounted for as the individual nucleotide. The results obtained in the investigation of four preparations of yeast ribose nucleic acid by the three different hydrolysis procedures described above are presented in Table II. Balances were also established to indicate recoveries in terms of nitrogen and of extinction at 260 *mμ*. The latter figures are of particular interest, since they show the recoveries from the hydrolysates of nucleic acid constituents with an absorption in the ultraviolet to have been almost complete. The pentose nucleic acid from pig pancreas (Preparation 5) also is described in Table II. The values given by Procedure 3 for this preparation as well as for Preparation 1 from yeast have been reported previously (9) and are included here for purposes of comparison. Table III summarizes the results obtained with the preparations of pentose nucleic acid from the livers of different animals. Conclusions as to the molar relationships between the nucleotide constituents are drawn in Table IV. These proportions are expressed as moles of guanylic, cytidylic, and uridylic acids per 10 moles of adenylic acid. The molar ratios of total purines to pyrimidines also are given.

The values submitted here represent the results of repeated determinations (usually six) on different hydrolysates. The figures for pyrimidine nucleotides afforded by Procedures 1 and 2 are reported without correc-

tion; those for purine nucleotides obtained by Procedure 1 carry a small correction. For the reasons given in the preceding paper (10) the values found for adenylic and guanylic acids were multiplied by a factor of 1.1. In two experiments (included in Table II), in which the chromatographic

TABLE II
Nucleotide Composition of Ribonucleic Acids of Yeast and Pig Pancreas

Preparation No *	Source	Hydrolysis procedure No.†	P accounted for as per cent of organic P in preparation				Balances		
			Guanylic acid	Adenylic acid	Cytidylic acid	Uridylic acid	P accounted for	N accounted for	Ultraviolet extinction accounted for‡
			per cent	per cent	per cent	per cent	per cent organic P in preparation	per cent N in preparation	per cent extinction of hydrolysate
1	Yeast	1	28.0	29.0	17.8	20.3	95.1	89.4	96.2
		2	28.8	25.8	16.5	19.5	90.6	86.0	
		3	25.6	26.1	24.4	8.3	84.4	82.4	
2	"	1	25.4	26.5	19.9	17.7	89.5	88.5	102.0
		2				18.8			
		3	25.4	24.4					
3	"	1	26.2	24.8	21.4	20.3	92.7	93.6	102.9
		1§	25.5	24.6	20.2	23.2	93.5	92.2	
		2	28.3	23.3	21.1	24.6	97.3	96.8	
4	"	3	25.9	24.2					101.0
		1	24.2	23.0	18.3	23.5	89.0	88.5	
		1§	23.4	23.2	17.5	24.1	88.2	87.4	
5	Pig pancreas	1	41.0	18.2	17.8	5.9	82.9	83.6	99.6
		2	40.7	18.9	17.8	8.4	85.8	85.6	
		3	40.2	16.6	20.5	4.6	81.9	82.1	

* The numbers refer to Table I.

† See the text for a definition of the hydrolysis procedures.

‡ The differences (Δ_{260}) between the extinction values of each nucleotide at 260 $m\mu$ and at 290 $m\mu$ are added and expressed as per cent of the Δ_{260} values determined for the hydrolysate before chromatographic separation (10).

§ In these analyses the "buffered system" (10) was used for separation.

separation had been carried out by means of the "buffered system" (10), only the adenylic acid values were so corrected.

The nucleic acid preparations from liver contained, as shown in Table I, varying amounts of desoxypentose nucleic acid. This contamination, slight in some cases, was quite considerable in the preparations from carcinomatous human liver; it was, in the analyses reported in Table III, taken into account in the following manner. The liberation of mononucleotides by mild alkaline hydrolysis, as practiced in Procedure 1, affects

only the pentose nucleic acids. Desoxypentose nucleic acids are degraded by alkali to a lower state of polymerization which, however, still is very far from that of a nucleotide. It has been confirmed in control experiments that the alkaline treatment of desoxypentose nucleic acids does not give rise to fractions that migrate from the starting point under the chromatographic conditions employed here. For this reason, the alkaline hydrolysis of a pentose nucleic acid, followed by the chromatographic

TABLE III

*Nucleotide Composition of Pentose Nucleic Acids from Liver of Different Species**

Preparation No.	Source	Hydrolysis procedure No.	P accounted for as per cent of organic P in preparation†									
			Guanylic acid		Adenylic acid		Cytidylic acid		Uridylic acid		Total	
			Found	Cor- rected	Found	Cor- rected	Found	Cor- rected	Found	Cor- rected	Found	Cor- rected
6	Pig	1	26.9	29.2	16.5	17.9	26.5	28.8	12.6	13.7	82.5	89.6
		2		33.9		19.5	28.4	30.9	17.0	18.5		103
		3		31.3		16.4						
7	Sheep	1	29.4	32.3	17.5	19.2	23.4	25.7	9.8	10.7	80.1	87.9
		3		34.8		19.9						
8	Calf	1	31.0	32.1	19.1	19.8	21.2	22.0	10.1	10.5	81.4	84.4
		3		31.1		19.0						
9	Beef	1	29.5	31.8	20.2	21.8	22.0	23.8	13.4	14.5	85.1	91.9
		3		32.9		21.5						
10	Human cancer, unaffected tissue	1	29.7	35.5	9.0	10.8	26.0	31.1	7.5	9.0	72.2	86.4
		3		38.8		12.1						
11	Human cancer, metastases	1	22.9	32.7	5.6	7.9	23.9	34.1	4.0	5.7	56.4	80.4
		3		38.6		9.4						

* See Table II for explanations.

† The corrected values reported for Procedure 1 were obtained by taking into account the desoxypentose nucleic acid contents of the preparations (see Table I). The figures given for Procedure 3 were obtained by purine analyses (5, 9) on both the pentose and desoxypentose nucleic acids from the same tissue specimen and by correction for the contribution made by the individual contaminant.

separation of the resulting pentose nucleotides, affords a fairly reliable picture of the proportional relationships of its constituents, regardless of the contaminating desoxypentose nucleic acid. The presence of the latter will, of course, depress the figures for recovered phosphorus, nitrogen, or absorbing material. In order to permit the comparison of recoveries for yeast and animal pentose nucleic acids, a set of values corrected for the presence of desoxypentose nucleic acid in the alkaline nucleic acid hydrolysates is included in Table III.

An entirely independent check on the validity of the ratios of guanylic

to adenylic acids found for the animal pentose nucleic acids is provided by the outcome of the purine analyses performed by Procedure 3. These analyses, based on the liberation of the purines by acid, will include the purines released by the contaminating desoxypentose nucleic acid. By purine analyses on both the pentose and the desoxypentose nucleic acids isolated from the same tissue specimen and by correction for the contribution made by the respective contaminant it was, however, possible to determine the purines derived from the pentose nucleic acid; these figures likewise are given in Table III.

TABLE IV
*Nucleotide Composition of Pentose Nucleic Acids; Molar Relationships**

Preparation No.	Source	Guanylic acid	Adenylic acid	Cytidylic acid	Uridylic acid	Purines Pyrimidines
1	Yeast	9.7	10	6.1	7.0	1.5
2	"	9.6	10	7.5	6.7	1.4
3	"	10.6	10	8.6	8.2	1.2
4	"	10.5	10	8.0	10.2	1.1
5	Pig pancreas	22.5	10	9.8	4.6	2.3
6	" liver	16.3	10	16.1	7.7	1.1
7	Sheep liver	16.8	10	13.4	5.6	1.4
8	Calf "	16.2	10	11.1	5.3	1.6
9	Beef "	14.6	10	10.9	6.6	1.4
	Carcinomatous human liver					
10	Unaffected tissue	32.9	10	28.8	8.3	1.1
11	Metastases	41.4	10	43.2	7.2	1.0

* Based on the results reported for Procedure 1 in Tables II to IV, adenylic acid being taken as 10. For Preparation 5 the value for uridylic acid found by hydrolysis by Procedure 2 was used.

Sugar Component of Liver Pentose Nucleic Acids

The identification of the sugar components by chromatography was carried out as described previously for the pentose nucleic acids of yeast and pig pancreas (9). The nucleic acid preparations from pig liver (Preparation 6), sheep liver (Preparation 7), calf liver (Preparation 8), and the two preparations from carcinomatous human liver (Preparations 10 and 11, Table I) were examined. The hydrolysis was performed in *N* sulfuric acid for 1 hour at 100°, 6.5 to 7.5 mg. of nucleic acid per cc. being employed. Portions of the hydrolysates (0.02 and 0.03 cc.) served for the chromatography on filter paper which was carried out in isobutyric acid (saturated with water) and in *n*-butanol-ethanol-water (4:1:5). In all chromatograms, which were developed by means of *m*-phenylenediamine (27), only

one sugar component was found; that occupied the position of *D-ribose*. The contaminating desoxypentose undoubtedly had been destroyed in the course of the hydrolysis.

TABLE V
*Cleavage of Pyrimidine Ribonucleosides and Ribonucleotides with Concentrated Formic Acid**

Experiment No.	Compound subjected to hydrolysis†	Duration of hydrolysis	Pyrimidine in compound hydrolyzed‡	Pyrimidine recovered					
				As nucleoside		As free pyrimidine		Total	
		hrs.	mg.	mg.	per cent	mg.	per cent	mg.	per cent
1	Cytidine	1	1.51	0.17	11	1.08	72	1.25	83
2	"	2	1.86	0.16	9	1.34	72	1.50	81
3	Cytidylic acid	1	0.73	0.35	48	0.34	47	0.69	95
4	" "	2	0.90	0.07	8	0.71	79	0.78	87
5	Uridine	1	1.71	0.69	40	1.02	60	1.71	100
6	"	2	1.91	0.61	32	1.37	72	1.98	104
7	Uridylic acid	2	0.80	0.56	70	0.16	20	0.72	90
8	" "	2	0.81	0.56	69	0.19	23	0.75	92
9	" "	2	0.86	0.29	34	0.41	48	0.70	82
10	" "	2	1.02	0.69	68	0.19	19	0.88	87
11	" "	2	1.30	0.68	52	0.44	34	1.12	86
12	" "	2	1.02	0.18	18	0.70	69	0.88	87
13	" "	2	1.09	0.21	19	0.74	68	0.95	87
14	" "	2	1.68	0.42	25	1.06	63	1.48	88
15	" "	2	2.05	0.28	14	1.59	77	1.87	91

* The nucleosides or nucleotides, containing the indicated amounts of pyrimidine, were heated to 175° in 0.5 cc. of concentrated formic acid in a bomb tube. The hydrolysates were adjusted to an accurate volume of 1 cc. and different aliquots subjected to chromatographic separation (5).

† The nucleosides used in Experiments 1, 2, 5, and 6 were commercial preparations: cytidine (Nutritional Biochemicals Corporation, Cleveland, Ohio); uridine (Schwarz Laboratories, Inc., New York). The preparation of cytidylic acid (Experiments 3 and 4) and additional hydrolysis experiments with this nucleotide have been described previously (9). In Experiments 7, 8, 9, and 11 diammonium uridylyte, whose preparation from cytidylic acid is described in the preceding paper (10), was used. In Experiment 10 a commercial sample of ammonium uridylyte was employed, in Experiments 12 to 15 a commercial preparation of uridylic acid; both came from Nutritional Biochemicals Corporation, Cleveland, Ohio.

‡ Cytosine in the case of cytidine and cytidylic acid, uracil in the case of uridine and uridylic acid.

Cleavage of Pyrimidine Nucleosides and Nucleotides with Concentrated Formic Acid

As an extension of previous studies on the hydrolysis of pentose nucleic acids by means of concentrated formic acid (9), additional experiments

were carried out on the cleavage by formic acid of the pyrimidine nucleosides and nucleotides. The bearing of these studies, which are summarized in Table V, on the problems with which the present paper deals will be discussed below. The chromatographic analysis of the hydrolysates was carried out as described previously (5) for the separation of pyrimidines. With *n*-butanol (saturated with water) as the solvent system, cytosine has an R_f value of 0.19, uracil of 0.32 (5). Under the same conditions the R_f of cytidine was found at 0.10, that of uridine at 0.18. The extracts of the separated components were prepared and read in the spectrophotometer in the usual fashion (5). The calculation of their contents from the spectrophotometric data followed procedures previously described (5, 10). If Δk_{\max} is defined as the difference between the specific extinctions of the substance at its absorption maximum and at $290 \text{ m}\mu$ (10), the values found in water were for cytidine (maximum $271 \text{ m}\mu$) 26.5, for uridine (maximum $262 \text{ m}\mu$) 39.9. The recovery of the nucleosides from test chromatograms was quantitative, 98 and 99 per cent of the uridine and cytidine, respectively, being found in the eluates.

DISCUSSION

The studies presented here show that it is necessary to draw a sharp distinction between the pentose nucleic acids of animal origin and those from yeast. All preparations of pentose nucleic acid from mammalian liver, regardless of the species from which they derive, appear to be characterized by a relatively high content in guanylic acid and, in most cases, also in cytidylic acid. This is most clearly shown in Table IV. Yeast ribonucleic acid, on the other hand, apparently is constructed according to entirely different principles.

A critical evaluation of the three methods used for the study of pentose nucleic acid composition can be based on the figures assembled in Tables II and III; it will lead to the conclusion that the most reliable values are yielded by Procedures 1 and 3 for purines, and by Procedures 1 and 2 for pyrimidines. The most convenient method for the study of the composition of pentose nucleic acids would appear to be Procedure 1. It is the only one which permits complete balances to be drawn, not only in terms of phosphorus and nitrogen, but also in terms of all nucleic acid components showing absorption in the ultraviolet region. The principal limitation of this method, already discussed in the preceding paper (10), may be seen in the fact that, since guanylic and uridylic acids are estimated in the same extract, a large excess of one of these nucleotides may render the value obtained for the other less accurate. In yeast ribonucleic acid, in which guanylic acid does not outweigh uridylic acid considerably, the figures yielded by the indirect (Procedure 1) and the direct determination (Pro-

cedure 2) of the latter are in good agreement (Table II). They are less so in those animal pentose nucleic acids that contain a very large excess of guanylic acid, and some of the uridylic acid values reported in Table III may, for this reason, be somewhat too low. In such cases the use of Procedure 2 seems preferable, at least as concerns the estimation of uridylic acid.

Procedure 3 is based on the release of the free purines by mild acid hydrolysis and of the free pyrimidines by an energetic treatment with concentrated formic acid. It has been applied to the analysis of pentose nucleic acids (9) and of desoxypentose nucleic acids (6-8). With respect to the latter, repeated studies on many different specimens have led to no important revision of the views set forth previously, though we hope to submit before long certain improvements and simplifications of the analytical procedures. As concerns the pentose nucleic acids, a comparison of the results presented in this paper will show that the purine values are practically unchanged, regardless of the method used, but there are discrepancies in the figures for pyrimidines. With Procedure 3, *i.e.* hydrolysis of the pyrimidine nucleotides to the free bases, much lower values for uracil and somewhat elevated values for cytosine were found. Furthermore, the total recovery of bases never accounted for more than about 84 per cent of the expected amount when pentose nucleic acids were examined by this procedure, in contrast to desoxypentose nucleic acids with which almost quantitative recoveries often could be recorded. This has already been pointed out and discussed at a previous occasion (9). These differences prompted a detailed study of the hydrolysis of pyrimidine ribonucleotides and ribonucleosides by concentrated formic acid, the results of which are summarized in Table V. This study also made necessary the chromatographic separation and spectrophotometric estimation of the ribonucleosides cytidine and uridine, and attention may be drawn to the inclusion of this work in the experimental part.

The results assembled in Table V demonstrate a great difference between cytidylic and uridylic acids and, to a lesser extent, between the corresponding nucleosides in their behavior on acid hydrolysis. Under the usual conditions of treatment with formic acid, cytidylic acid (Experiment 4) yielded 79 per cent of its cytosine content as the free pyrimidine, 8 per cent as cytidine. These figures are in good agreement with previous hydrolysis experiments (9). Cytidine (Experiment 2) was split to about the same extent. In both cases, more markedly with the nucleoside, there was evidence that some degradation beyond the pyrimidine stage had taken place, leading to a small quantity of products with no, or diminished, absorption in the ultraviolet. No indication of the formation of uracil was found. Uridylic acid not only proved much more refractory to hydrolysis,

but the results varied with the preparations employed. When samples of ammonium uridyate were subjected to hydrolysis, almost two-thirds of the reaction product was found to consist of the nucleoside uridine, the rest of uracil (Experiments 7 to 11). When free uridylic acid was examined, a larger proportion of the uracil was split off as the free pyrimidine (Experiments 12 to 15). Uridine itself also proved very resistant (Experiment 6). The reasons for this behavior are not quite clear. It is possible that the primary products of the acid cleavage of a pyrimidine nucleotide, and especially of uridylic acid, can be either ribose phosphoric acid and pyrimidine or phosphoric acid and nucleoside. Since the latter is split further at a slow rate, the extent to which nucleoside was formed initially will influence the outcome of the experiment. It is, moreover, not unlikely that not only yeast adenylic acid, as has been shown already (25, 26), but also the other ribonucleotides, occur in several isomeric forms, whose relative concentrations in a nucleotide preparation or in a nucleic acid hydrolysate may influence the manner in which further cleavage occurs.

These findings serve to explain the analytical differences, as regards the pyrimidines, between Procedures 1 and 3. In the latter arrangement only a portion of the uridylic acid present was cleaved to uracil. The remainder was converted to uridine, which, in the butanol-water system used, migrated to a position on the chromatogram very close to that of cytosine. The figures for uracil, obtained in this manner, consequently were too low, those for cytosine too high.

It now remains to offer a brief discussion of the ribonucleotide composition of the various pentose nucleic acids that form the subject of the present study. Their elementary composition is summarized in Table I, in which some of their optical characteristics are also compared.⁶ It will be seen that contamination with desoxypentose nucleic acid was not considerable, except in Preparations 10 and 11 from carcinomatous human liver. The presence in a pentose nucleic acid specimen of the desoxy compound is, with the methods of analysis at present available, of much less concern than the converse. One particular advantage of the study of the nucleotide composition of pentose nucleic acids may be seen in the fact that it is not affected by the presence of desoxypentose nucleic acid, since the latter is not degraded to mononucleotides by alkali. Attention should

⁶ One gains the impression that the extinction intensity of a nucleic acid preparation at its absorption maximum is in inverse relationship to its molecular size. The very highly polymerized desoxypentose nucleic acids examined in this laboratory had an $\epsilon(P)$ around 6400 (6, 7, 23). The $\epsilon(P)$ of the pentose nucleic acids of much lower molecular weight studied here varied from 8500 to 10,000; and this value increased considerably on hydrolysis of the preparation to the mixture of component mononucleotides. We shall return to this point in a forthcoming publication in connection with a discussion of enzymatic and hydrolysis experiments.

be directed to the recovery balances drawn in all tables, in greatest detail in Table II. They show that the recovery in terms of extinction, *i.e.* of nucleic acid components absorbing in the ultraviolet region, was practically quantitative, that in terms of phosphorus and nitrogen about 10 per cent lower. This indicates a small amount of contamination with substances other than nucleotides.

The composition of four preparations of yeast ribonucleic acid is presented in Tables II and IV. The two purine nucleotides were present to about the same extent and in almost equimolar proportions in all samples. A slightly greater fluctuation was shown by the pyrimidine nucleotides. It would be premature to draw conclusions from these differences. It is, perhaps, significant that the specimens prepared in the laboratory (Preparations 3 and 4) had a relatively higher pyrimidine content than the purified commercial preparations, No. 1 and 2. For the rest, a more detailed discussion would have to repeat much of what has already been said (9). The same also holds for the pentose nucleic acid preparation from pig pancreas (Preparation 5) which has been considered previously (9). If the more reliable uridylic acid figure found by Procedure 2 is used (Table II), *i.e.* 0.084 mole of uridylic acid per mole of phosphorus, the molar proportions of nucleotides are identical for both Procedures 1 and 2. For 1 mole of uridylic acid there were found in the pancreas preparation 4.9 moles of guanylic acid, 2.2 moles of adenylic acid, and 2.1 moles of cytidylic acid. In view of the lability of pancreas ribonucleic acid often described (for a recent demonstration, see (28)), a comparison with recently published analytical figures (29) appears pointless, except that these, too, show the relatively high concentration of guanylic acid.

Whereas the high guanylic acid content of pancreas nucleic acid has been commented upon frequently (see the survey in (9)), it now appears from the experiments reported here that animal pentose nucleic acids in general are distinguished from those of yeast by high concentrations of this nucleotide. The preparations from pig, sheep, and ox liver all had about 60 per cent more guanylic acid than adenylic acid. Cytidylic acid also was present in large amounts and was followed by adenylic and uridylic acids, the latter definitely a minor component. To attempt a wide generalization at this early stage of the investigations appears hazardous. This applies to an even higher degree to the two preparations from carcinomatous human liver which were characterized by a remarkable preponderance of guanylic and cytidylic acids, present in almost equimolar proportions. Whether this proportional correspondence, also observed in other preparations (*e.g.* Preparation 6 from pig liver), is more than accidental, will, perhaps, appear from further work.

In the course of this work it has been possible to demonstrate the presence of ribose in the hydrolysates of all nucleic acids studied here. This

sugar has already been identified previously as a component of one liver pentose nucleic acid; *viz.*, that from sheep liver (16).

The application of the methods described in this and in the preceding paper (10) to the study of the course of hydrolysis of different ribonucleic acids by alkali and by ribonuclease will be presented in a subsequent publication.

SUMMARY

Preparations of ribonucleic acids derived from yeast, pig pancreas, and the liver of pig, sheep, ox, and man (including carcinomatous tissue) were hydrolyzed by a variety of methods, and the nucleotide composition was determined. In contrast to yeast ribonucleic acid, all preparations of pentose nucleic acids from animal tissues were characterized by a high proportion of guanylic acid and, in most of the liver preparations, also of cytidylic acid.

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PROTEOLYTIC ACTIVITY DETERMINED WITH A SUBSTRATE TAGGED WITH RADIOACTIVE IODINE*

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The proteolytic enzyme, plasmin, is occasionally found in the plasma of patients with a variety of pathologic conditions. Its presence is detected by observing the complete dissolution of the clot of fibrin (fibrinolysis) formed by the coagulation of blood (1). Methods based on time of fibrinolysis are unsuitable for detecting quantities of plasmin that are insufficient to produce complete lysis of the clot. The method described in this communication was developed to increase the sensitivity of the fibrinolytic test and to establish it on a quantitative basis. The method consists, essentially, of using fibrin tagged with radioactive iodine as a substrate and of relating increases of radioactivity in the non-clottable protein fraction to proteolytic activity.

Materials and Methods

The following reagents were used.

Thrombin—A commercial preparation called Topical Thrombin, produced by Parke, Davis and Company (2).

Trypsin—A crystalline trypsin preparation, obtained from the Plaut Research Laboratory. It contained approximately 50 per cent magnesium sulfate.

Plasmin¹—A preparation from bovine plasma prepared by the method of Loomis *et al.* (3).

Fibrinogen—Fraction I from bovine plasma² (4) was further purified by the method of Ware, Guest, and Seegers (5). The clottable nitrogen of the final preparation constituted at least 88 per cent of the total nitrogen. The fibrinogen was kept frozen at -10° as a 1 to 2 per cent solution in

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¹ Kindly supplied by Dr. Eugene Loomis.

² Supplied by Armour and Company.

0.01 M phosphate buffer in physiological saline containing 0.1 per cent sodium citrate at pH 7.5.

Iodination of Fibrinogen—The radioactive iodine used was I^{131} with a half life of 8 days obtained as a carrier-free sodium iodide solution. 1 mc. was equivalent to approximately 10^{-8} gm. of iodine. The iodinating solution was prepared as follows: To 1 mc. of radioactive iodine in 0.25 to 0.5 ml. of water were added 0.6 ml. of 0.002 M sodium iodide and 0.6 ml. of 0.02 M sodium nitrite. The solution was acidified to below pH 4 by the addition of 0.5 N hydrochloric acid. After a few minutes the solution was neutralized by the addition of 0.25 N sodium hydroxide and diluted to a volume of 25 ml. with M/15 phosphate buffer, pH 7.5.

The iodinating solution was added drop by drop with continuous stirring over a 10 minute period at room temperature to 25 ml. of approximately 1.4 per cent fibrinogen solution. The mixture was kept at room temperature for 10 minutes and then dialyzed against 0.01 M phosphate buffer in physiological saline containing 0.1 per cent sodium citrate at pH 7.5. Dialysis was carried out at 4° against a total of 10 liters of buffer for 2 days, with 5 liters of buffer each day. The dialyzing bag was rotated continuously by means of an electric motor.

Following dialysis, the solution was heated to 37° to dissolve the fibrinogen that partly precipitated in the cold and was centrifuged at 2000 r.p.m. for several minutes to eliminate a small amount of undissolved protein. The iodinated fibrinogen stock solution was stored in small lots at -10°.

Properties of Fibrinogen Tagged with Radioactive Iodine—The clottable fraction of the fibrinogen solution took up approximately 5 per cent of the total radioactive iodine used in the iodination. This corresponds roughly to 1 atom of I^{131} for 180,000 molecules of fibrinogen and 1 atom of carrier iodine for 12 molecules of fibrinogen. After dialysis, the clottable fraction contained approximately 75 per cent of the total radioactivity of the iodinated fibrinogen solution. This proportion fluctuated between 70 and 80 per cent for different batches but remained constant for each batch during the period of storage (6 weeks). The proportion remained unchanged after incubation of the solution for 1 to 2 hours at 37° over the range, pH 6.3 to 8.7, as well as after a 10-fold dilution. The proportion of radioactivity contained in the clottable fraction of the iodinated fibrinogen solution could not be increased by dialysis prolonged beyond the 2 day period.

The clottable nitrogen of the iodinated fibrinogen solution did not vary significantly during the period of storage, indicating that the amount of iodine used in these experiments did not alter the clottability of fibrinogen by thrombin.

Little or no uptake of radioactive iodine occurred when no carrier iodine was used. However, when the carrier iodine was increased to 20 times the amount used in these experiments, the fibrinogen solution became unstable and showed increasing precipitation on storage. When the amount was increased to 220 times, the fibrinogen precipitated completely in 20 minutes. The amount of radioactive iodine used was very small compared to the amount of non-radioactive iodine, the ratio of the 2 being approximately 1:15,000. It was, therefore, possible to increase the radioactivity in the fibrinogen by increasing the proportion of I^{131} to carrier iodine without significantly altering the total iodine content of the preparation.

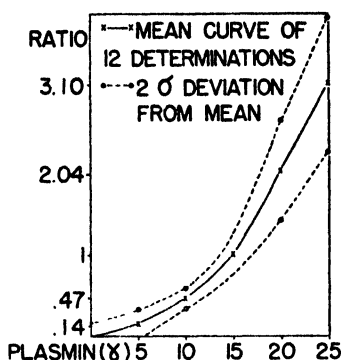


FIG. 1

FIG. 1. Proteolysis of iodinated fibrinogen by plasmin. Reaction carried out at pH 7.4. Substrate, 0.08 per cent iodinated fibrinogen solution. Thrombin, 1 ml. = 100 units. Total volume in each tube, 1.1 ml.; temperature 37°; incubation time, 50 minutes.

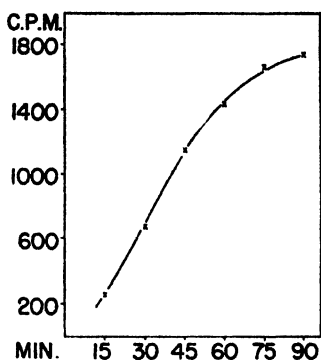


FIG. 2

FIG. 2. Proteolysis of iodinated fibrinogen by plasmin. Conditions as in Fig. 1, except that 0.12 per cent iodinated fibrinogen solution is the substrate. Thrombin, 1 ml. = 50 units.

Determination of Proteolytic Activity—The reaction was carried out in test-tubes (10 × 70 mm.) immersed in a constant temperature water bath. The stock iodinated fibrinogen solution was diluted with buffer to a concentration of 0.08 per cent. This constituted the substrate. For measurement of proteolytic activity, varying amounts of a plasmin solution were added to 0.5 ml. of substrate. The total volume of the reaction mixture was kept constant by the addition of suitable amounts of buffer solution. Coagulation was produced in each tube by adding 0.1 ml. of thrombin and stirring with a glass rod. A solid clot formed within 15 seconds after the addition of thrombin. After incubation, the supernatant solution of the partly digested clot was separated by breaking the clot with a glass rod and filtering through Whatman No. 1 paper. Filtration was completed

in 1 minute. Measurement of β particles was made on a 0.5 ml. aliquot of each filtrate, which was placed in a suitable container for the Geiger counter, made alkaline with 0.1 ml. of 0.1 N sodium hydroxide, and dried before counts per minute were determined.

Results

Plasmin Activity As Measured By Radioactivity of Non-Clottable Fraction of Substrate—Varying amounts of plasmin were incubated with a constant amount of substrate and the radioactivity in the supernatant solution was measured. The same experiment was repeated twelve times in three groups of four determinations. In every experiment the number of counts per minute obtained with 0.015 mg. of plasmin was arbitrarily selected as

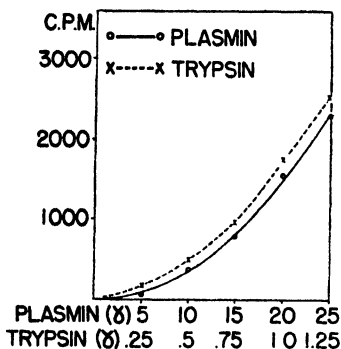


FIG. 3

FIG. 3. Comparison of proteolysis of iodinated fibrinogen by plasmin and trypsin. Conditions as in Fig. 1.

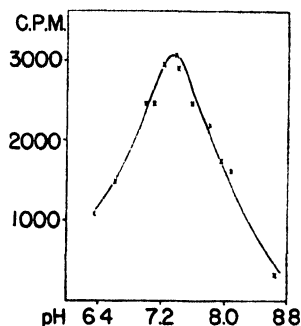


FIG. 4

FIG. 4. The effect of pH on proteolysis of iodinated fibrinogen by plasmin. Conditions as in Fig. 1; pH varied by means of phosphate buffer.

a reference value. For every experiment, a ratio was made of the counts obtained with each amount of plasmin to the reference value. The mean ratio for each amount of plasmin and the 2σ variation of the ratio were determined. The results are shown in Fig. 1. By using a ratio of counts per minute, the results obtained for substrates containing different amounts of radioactivity are comparable and the graph may be considered a standard curve with standard variations representing the action of plasmin as measured by radioactivity. Larger amounts of plasmin could be measured in a similar fashion by increasing the amount of substrate.

Effect of Time of Incubation As Measured by Radioactivity of Non-Clottable Fraction of Substrate—When a constant amount of plasmin was incubated with a constant amount of substrate for varying periods of time, radioactivity in the non-clottable fraction increased with time. The re-

sults, representing the average values of three experiments, are shown in Fig. 2. The enzyme activity was found to increase at a constant rate for at least 50 minutes.

Comparison of Plasmin and Trypsin Activity—For purposes of comparison, varying amounts of plasmin and crystalline trypsin were incubated with a constant amount of substrate. The results, representing the average values of five experiments, are shown in Fig. 3. The curves were parallel. 25 mg. of the trypsin preparation used produced 0.1137 mg. of tyrosine in 10 minutes at 25° when assayed by the method of Anson and Mirsky (6). By comparing these results with those of Fig. 1 it was determined that increments of plasmin activity equivalent to 2.5×10^{-9} to 4.7×10^{-9} hemoglobin trypsin units could be measured.

Effect of pH—The optimal pH of the reaction was found to be 7.4 by this method. Below pH 6.3 the substrate precipitated, and above pH 8.7 the clotting time with large amounts of thrombin was longer than 1 hour. The pH curve obtained by this method (Fig. 4) was identical with the curve obtained by conventional methods.

Effect of Additional Amounts of Iodine and Iodide in Enzyme-Substrate Reaction Mixture—No effect on the reaction rate was observed when molecular iodine was added to the reaction mixture in a concentration 40 times that used in iodination or when iodide in the form of sodium iodide was added in a concentration 1000 times greater than the amount of iodine used in iodination.

DISCUSSION

Radioactive iodinated fibrinogen was easily prepared and could be used for a period of at least 6 weeks when stored in the cold. Plasmin and trypsin were tested on the clotted radioactive substrate and the data indicate that increases of radioactivity (as measured by β -ray counts) in the supernatant solution of the clot can be taken as a measure of enzyme activity. The amount of iodine present in the experimental preparation did not appear to modify the activity of the two proteolytic enzymes tested by this method. Partial precipitation of the substrate at pH 6.3 and failure of clot formation with large amounts of thrombin at pH 8.8 limited the measurement of enzyme activity to this range of pH. The amount of radioactivity in the tagged substrate was small and did not constitute a health hazard if a few simple precautions were taken.

Several methods have been proposed for measurement of plasmin and antiplasmin in plasma and plasma derivatives. The most common method used is based on a measurement of time for complete dissolution of a standard fibrin clot by the enzyme (7). This method has two limitations: first, the end-point is somewhat uncertain, especially when the time of

dissolution is prolonged; and second, amounts of enzyme which are too small to digest the clot completely cannot be measured because no end-point is reached.

The method described herein does not have these limitations. It permits measurement of amounts of enzyme that do not completely digest the clot and is based on the measurement of end-products of the reaction between plasmin and fibrin rather than on a measurement of time. The end-products are defined by the fact that they are not clottable by thrombin. Since these end-products are radioactive, they can be measured accurately and simply by a Geiger counter without the use of chemical or colorimetric techniques.

The sensitivity of the method depends in great part upon the use of thrombin as the agent for precipitating protein. In contrast to most protein precipitants used in proteolytic enzyme work, as for instance trichloroacetic acid, thrombin acts specifically in precipitating only fibrinogen by the formation of fibrin. The early split-products of fibrin which would be precipitated by reagents such as trichloroacetic acid are soluble in the presence of thrombin. The usefulness of such a highly specific protein precipitant as thrombin is especially apparent when the enzyme plasmin is used, since it is known that the products of plasmin digestion consist mostly of large molecular fragments that still have the characteristics of proteins (8). Another factor in the sensitivity of the method is the measurement of reaction products by the radioactivity they contain instead of by a colorimetric tyrosine measurement. The blank in the radioactive method depends solely upon the substrate and is not contributed to by the other reagents used. When the tyrosine method is used for the measurement of plasmin activity in plasma or plasma derivatives, the color-producing material in the reagents may make the value of the blank so high that experimental variations due to enzyme action become relatively insignificant.

SUMMARY

Fibrinogen was tagged with radioactive iodine by a simple method. A fibrin clot formed from the tagged fibrinogen was found to constitute an adequate substrate for certain proteolytic enzymes within the range, pH 6.3 to 8.8. The increase of radioactivity in the filtrate of this clot after incubation with enzyme was taken as a measure of proteolytic activity. In this way it was possible to measure accurately increments of proteolytic activity equivalent to 2.5×10^{-9} to 4.7×10^{-9} hemoglobin trypsin units. The method was more sensitive and more accurate than methods used previously for the detection and measurement of small amounts of plasmin and trypsin and may be applicable to other similarly reacting proteolytic enzymes.

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STUDIES ON HIGH POTENCY OXYTOMIC MATERIAL FROM BEEF POSTERIOR PITUITARY LOBES*

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Recently it was reported from this laboratory that the Craig counter-current distribution technique can be utilized in the purification of the oxytomic principle of the posterior lobe of the pituitary (1). An oxytomic concentrate obtained from Parke, Davis and Company had served as the source material. The most potent preparation obtained from this concentrate had an oxytomic activity of approximately 850 units per mg. It gave a counter-current distribution curve suggesting that the material was nearly pure or that, if any impurity was present, it possessed a distribution coefficient almost identical with that of the oxytomic factor. The amino acid composition of a hydrolysate of this preparation was then determined by Pierce and du Vigneaud (2) by application of the technique developed by Moore and Stein (3-6), in which small amounts of amino acids are separated by chromatography on starch columns and determined by a quantitative ninhydrin reaction. The hydrolysate was found to contain leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, ammonia, and cystine, with exceedingly slight traces of other amino acids. Furthermore, within the error of the experiment, the molar ratios of these components were 1:1 with the exception of that of ammonia and tyrosine; the latter may have been partially destroyed during hydrolysis.

Because of the unavailability of further supplies of the earlier concentrate for the extension of this work, we have now developed a method for obtaining a high potency oxytomic preparation in which lyophilized beef posterior pituitary lobes¹ serve as the starting material. Preparations with potencies of 700 to 800 units per mg. have been made routinely from the lyophilized lobes by a combination of the procedure of Kamm *et al.* (7) for the separation of oxytomic and pressor activities and that of Livermore and du Vigneaud (1) for the concentration of the oxytomic principle. The material prepared by this method possessed the same distribution

* The authors wish to express their appreciation to the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this investigation.

¹ The authors are indebted to Eli Lilly and Company, who generously made the lyophilized posterior pituitary lobes available.

coefficient between *sec*-butyl alcohol and 0.05 per cent acetic acid, namely 0.4, as did the oxytocic material isolated from the commercial concentrate.

Sufficient material thus became available to make a thorough accounting of the nitrogen, sulfur, and carbon content of the high potency oxytocic preparation in terms of the amino acids therein.

EXPERIMENTAL

Posterior Pituitary Material—The posterior lobes were lyophilized beef posterior pituitary lobes,¹ which were extracted twice with 500 ml. portions of acetone in a Waring blender. The acetone-insoluble material contained 1.5 units of oxytocic activity per mg. when assayed by the chicken blood pressure method of Coon (8). This assay procedure was used throughout the present work.

TABLE I

Preparation by Procedure of Kamm et al. (7) of Oxytocic Material with Potency of 7 to 10 Units per Mg.

Steps in procedure	Weight	Total units oxytocic activity
	gm.	
Acetone-extracted posterior lobes	100	149,000
0.25% acetic acid extract (volume, 11 liters) . .		132,000
Extract concentrated to 1 liter <i>in vacuo</i> at 40° .		116,000
Ammonium sulfate ppt.	68	72,000
Material extracted by glacial acetic acid and then pptd. by ether and hexane	9	62,000

Preparation of Material Having Potency of 7 to 10 Units of Oxytocic Activity per Mg.—Table I shows the results of preparation of material of 7 to 10 units per mg., according to the procedure of Kamm *et al.* (7). The weight from 100 gm. of posterior lobes was 9 gm. of powder with an activity of 6.9 units per mg. Most preparations had a lower weight but about the same number of total units of activity. A total of 866 gm. of lyophilized posterior lobes was carried through this procedure in about 100 gm. portions. The yield was 53 gm. of powder with an oxytocic activity of 7 to 10 units per mg. This powder was stored *in vacuo* over phosphorus pentoxide.

Extraction of Oxytocic Activity with sec-Butyl Alcohol—Preliminary experiments indicated that no appreciable amount of oxytocic activity could be extracted from a 0.25 per cent acetic acid extract of the posterior lobes by *sec*-butyl alcohol. The following experiment was carried out on material of 7 to 10 units per mg. 50 mg. of powder were dissolved in 2.5 ml. of 0.05 per cent acetic acid saturated with *sec*-butyl alcohol, and the solu-

tion was extracted six times with 2.5 ml. portions of *sec*-butyl alcohol saturated with 0.05 per cent acetic acid. The butyl alcohol phase was diluted with an equal volume of water and lyophilized. The material was dissolved for assay in 1 ml. of 0.05 per cent acetic acid and the assays showed that only about 10 per cent of the activity was extracted by the *sec*-butyl alcohol. Essentially the same results were obtained in two repetitions of the experiment.

Since the oxytocic activity could not be extracted at this stage, 1 gm. of the powder of 7 to 10 units of oxytocic activity per mg. was treated as described by Kamm *et al.* (7) for the separation of oxytocic activity from the pressor activity. This procedure consists of the solution of the material of 7 to 10 units per mg. in 98 per cent acetic acid, and precipitation with ether of the pressor activity together with half of the oxytocic activity. The remaining half of the oxytocic activity is in the ether-acetic acid filtrate and is precipitated as an oil by the addition of hexane. After lyophilization, the oil yielded 78 mg. of solids with a total activity of 2500 units. This was dissolved in 10 ml. of 0.05 per cent acetic acid and extracted six times with 10 ml. portions of *sec*-butyl alcohol as described in the preceding paragraph. It was found that 95 per cent of the activity had been extracted by the *sec*-butyl alcohol. This result was also confirmed on repetition of the experiment. This change in extractability from an aqueous solution by *sec*-butyl alcohol will bear further investigation as to its full significance.

Preparation of Material of 250 to 300 Units per Mg. from Material of 7 to 10 Units per Mg.—The following procedure, based on the results described in the previous section, has been used for the preparation of material of 250 to 300 units of oxytocic activity per mg. 10 gm. of the powder of 7 to 10 units per mg. are treated by the procedure of Kamm *et al.* (7) for the separation of pressor and oxytocic activities. The oil resulting from the precipitation of the oxytocic activity by hexane is taken up in 25 ml. of water. This is extracted with *sec*-butyl alcohol and the extract is washed with 2 M phosphate buffer, dried, concentrated, and lyophilized, as described by Livermore and du Vigneaud (1). Care must be taken during lyophilization to make certain that the material is completely frozen, or inactivation may occur. Also, if it is necessary to interrupt the lyophilization, the material should be melted and refrozen, since storage of partially dry material, even in the cold, may cause some inactivation.

The yield from the 53 gm. of material of 7 to 10 units per mg. was 242,000 units of oxytocic activity. The material had a potency of 250 to 300 units per mg. It was dissolved in 0.05 per cent acetic acid (5 mg. per ml.) and stored at -20° until it was subjected to the Craig counter-current distribution technique (9).

Preparation of Material of 700 to 800 Units per Mg. from Material of 250

to 300 Units per Mg.—The method of distribution between *sec*-butyl alcohol and 0.05 per cent acetic acid described by Livermore and du Vigneaud (1) was used. First, 53 transfers were applied to several batches of material of 250 to 300 units per mg. and then the material from the tubes containing the peak of activity in the 53 transfer distributions was carried through 100 additional transfers. The ten tubes representing the peak of

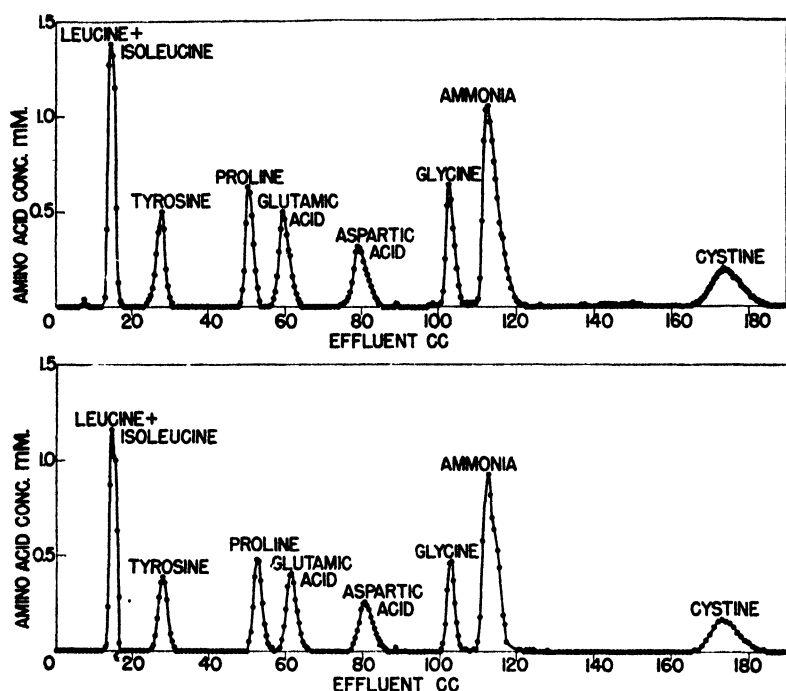


Fig. 1. Separation of amino acids from the hydrolysate of 1.952 mg. of high potency oxytocic material (upper curve) and from 1.80 mg. of an artificial mixture of amino acids (lower curve) simulating the amino acid composition of the oxytocic material. Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 *N* HCl followed by 2:1 *n*-propyl alcohol-0.5 *N* HCl. Column, 13.4 gm. of starch (anhydrous); diameter, 0.9 cm.; height, approximately 30 cm.

the activity from the 100 transfer distribution contained approximately 60 mg. of solids which possessed a total activity of 40,000 units. This was obtained from 400 mg. of starting material with an activity of 250 to 300 units per mg. The most active fraction from the 100 transfer distribution had a potency of approximately 800 units per mg. This material possessed the same distribution coefficient (0.4) between *sec*-butyl alcohol and 0.05 per cent acetic acid as did the preparation of Livermore and du Vigneaud. After assay, the active fractions were lyophilized from a volume of 2 ml.

of 0.05 per cent acetic acid in test-tubes with constricted necks. The tubes were then sealed while the material was still *in vacuo*, and stored at -20° .

Analysis of High Potency Material—The lyophilized fractions from six tubes containing the maximum activity from the 100 transfer distribution were combined. This material, consisting of 39 mg., was utilized for ele-

TABLE II

Amino Acid Composition of High Potency Oxytocic Material Prepared from Beef Posterior Pituitary Lobes

The values are from the chromatograms given in Fig. 1.

Amino acid	Mg. amino acid per mg. oxytocic material*	Mg. amino acid residue per mg. oxytocic material*	Mg. nitrogen per mg. oxytocic material*	Molar ratio with leucine expressed as 1	Mg. amino acid per mg. total artificial mixture placed on column	Mg. amino acid per mg. total artificial mixture recovered from column
Leucine	0.115†	0.099	0.0123	1.00	0.103	0.0955
Isoleucine	0.115†	0.099	0.0123	1.00	0.102	0.0955
Tyrosine	0.154	0.139	0.0119	0.97	0.140	0.133
Proline	0.102	0.086	0.0124	1.02	0.079	0.084
Glutamic acid†	0.142	0.125	0.0135	1.10	0.119	0.118
Aspartic acid†	0.127	0.110	0.0133	1.09	0.107	0.101
Glycine	0.071	0.050	0.0132	1.09	0.050	0.0499
Ammonia	0.049	0.049	0.0401	3.28	0.130§	0.130§
Cystine	0.227	0.210	0.0265	1.08	0.168	0.184
Total		0.967	0.155		0.998	0.991

* The values are expressed in relation to unhydrolyzed oxytocic material and are corrected for moisture.

† The values for leucine and isoleucine on a chromatogram with a solvent mixture of 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water (3) are 0.127 and 0.120, respectively.

‡ The glutamic acid and aspartic acid values are corrected for being 7 and 6 per cent low, respectively (5).

§ Expressed as ammonium chloride. The value of 0.130 in the recovery from the column is included only for purposes of calculating the total recovery. No calculation could be made of the actual amount recovered because of temporary failure of the fraction collector (3) to operate

mentary analyses and for the determination of the amino acid composition. The following analytical data (corrected for moisture content) were obtained: C 50.12 per cent, H 6.84 per cent, N 16.12 per cent (micro-Kjeldahl), S 6.15 per cent (micro-Carius), and ash 0.11 per cent. The moisture content (determined by drying the sample *in vacuo* at 100°) was 9.65 per cent at the time the material was weighed for amino acid analysis.

Amino Acid Composition of Hydrolysate of High Potency Material—9.902

mg. of the material described in the previous section were dissolved in 2 ml. of 6 *N* hydrochloric acid which had been distilled twice in glass, and heated under a reflux in an atmosphere of nitrogen for 16 hours at a bath temperature of 132°. The amino acid composition of the hydrolysate was determined by chromatography on starch columns exactly as described before (2). With a solvent mixture of 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1 *N* HCl followed by 2:1 *n*-propyl alcohol-0.5 *N* HCl, exactly the same spectrum of amino acids was obtained as that given (2) by the material prepared by Livermore and du Vigneaud (1). Fig. 1 shows the chromatogram of the hydrolysate of the oxytomic material, along with the chromatogram of an artificial mixture of leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, ammonia, and cystine.²

Upon resolution of the peak for leucine plus isoleucine with the solvent mixture described previously (3), it was found to consist of an equimolar mixture of leucine and isoleucine, as in the previous case (2). The peak at the glutamic acid-alanine position was also shown to consist almost solely of glutamic acid by the procedure of Stein and Moore for the separation of glutamic acid and alanine (5).

Table II presents the quantitative recovery of the amino acids from the chromatogram shown in Fig. 1. The recovery is expressed as mg. of amino acid, amino acid residue, and nitrogen per mg. of unhydrolyzed oxytomic material. The molar ratios of the components to each other are given, with that of leucine arbitrarily chosen as 1. The composition of the artificial mixture placed on the column and the recovery of each amino acid are also given. In the case of the artificial mixture, it may be noted that 0.168 mg. of cystine per mg. of artificial mixture was placed on the column and that 0.184 mg. was recovered.

DISCUSSION

From the data in Fig. 1 and Table II it can be seen that the hydrolysate of the oxytomic material prepared from lyophilized posterior lobes of the pituitary gland contains leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, ammonia, and cystine with only slight traces of any other amino acid. It is extremely significant that the same spectrum of amino acids was obtained from this preparation as was obtained from the material prepared from the earlier oxytomic concentrate (2). However, the tyrosine value was somewhat higher and the ammonia value lower than the values obtained previously, presumably because the hydrolysis of the high potency preparation in the present case was carried out under an atmosphere of nitrogen. With the change in the tyrosine value, the molar ratio of the amino acids to one another is more clearly 1:1 and

² The artificial mixture was made from specially purified amino acids kindly provided by Dr. William H. Stein and Dr. Stanford Moore.

that between any one amino acid and ammonia is 1:3 within experimental error. Hydrolysis under nitrogen also resulted in a higher value for cystine than that obtained previously. In terms of amino acid residues and ammonia, the analysis accounted for 96.5 per cent of the nitrogen of the unhydrolyzed compound. Furthermore, the cystine content accounted for all of the sulfur.

We would like to point out the close agreement of the experimentally determined values of carbon, hydrogen, nitrogen, and sulfur and the values calculated for a polypeptide composed of 1 mole of each of these eight amino acids and 3 moles of ammonia. If the hormone were a polypeptide of this composition, with the amino acids in peptide linkage and the ammonia in amide linkage, one would have in the molecule two free amino groups and one free carboxyl group. On this basis, the molecular weight³ would be 1025 and the following percentage composition would obtain: C 50.4, H 6.68, N 16.40, and S 6.25. The values found were C 50.12, H 6.84, N 16.12, and S 6.15.

As yet there is no evidence that the oxytocic hormone, if it is a polypeptide, is a simple straight chain polypeptide. It could well be a more complicated one with some type of ring structure or branched polypeptide structure. Furthermore, it is possible that a very small moiety or moieties, not detected by the means so far employed, could be present. Nevertheless, we felt that this correlation between experimental and calculated values on the basis outlined was worthy of note.

Finally it should be mentioned that, although it seems extremely likely from the data so far obtained from counter-current distribution studies and from amino acid analysis that the hormone is composed of these eight amino acids and ammonia, the data do not preclude the possibility of the existence in this high potency preparation of other compounds of the same amino acid composition and distribution coefficient.

The authors wish to express their appreciation to Dr. William H. Stein and Dr. Stanford Moore for their valuable advice during the course of this investigation. The authors also wish to acknowledge the assistance of Mrs. Jacqueline Everett Parton and Mrs. Elizabeth Copeland Pierce and to thank Dr. J. R. Rachele and Mrs. Josephine T. Marshall for the microanalyses.

³ A preliminary molecular weight determination, kindly carried out for us by Dr. Paul H. Bell, Stamford Research Laboratories, American Cyanamid Company, with a thermoelectric osmometer method (10), indicated a molecular weight in the range of this minimum molecular weight. Values of 928 and 802 were obtained on samples similar to those described. Oxidized glutathione was used as a model compound and gave a value of 615 (calculated 612). Further studies of the molecular weight will be undertaken.

SUMMARY

A method of preparation of oxytomic material of approximately 800 units per mg. from lyophilized beef posterior pituitary lobes has been presented.

The distribution coefficient and the amino acid pattern of this high potency preparation have been found to be identical with those of another preparation reported previously. The amino acids leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, and cystine were shown to be present in a 1:1 ratio. The molar ratio of any amino acid to the ammonia found was 1:3.

In terms of amino acid residues and ammonia, the analysis accounted for 96.5 per cent of the nitrogen of the unhydrolyzed compound. Furthermore, the cystine content accounted for all of the sulfur.

The possible interpretation of these findings has been discussed.

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THE NET UTILIZATION OF AMMONIUM NITROGEN BY THE GROWING RAT*

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The possibility that monogastric animals might utilize ammonium nitrogen was indicated by the experiments of Foster, Schoenheimer, and Rittenberg (1), who observed a rapid incorporation of dietary ammonium nitrogen containing N^{15} into rat tissue proteins. A study was therefore made of the ability of ammonium nitrogen to replace the non-essential amino acids in the diet of the growing rat. A preliminary report of the results was made under this title some time ago (2). Growth and nitrogen balance data indicated that, in the young rat fed a diet containing only the ten essential amino acids (3), dietary ammonium nitrogen was retained and it supported as good growth as did a supplement of a mixture of eight non-essential amino acids containing an equivalent amount of nitrogen. Although our work on this problem was undertaken independently and the preliminary publication was presented prior to the announcement (4) of similar work with urea and glycine in Professor W. C. Rose's laboratory, the present paper is in many respects a confirmation of the data since reported in detail by Rose, Smith, Womack, and Shane (5). In addition, some work is reported on the attempted replacement of certain essential amino acids and on the D-amino acid oxidase activity of the tissues of rats fed various amino acid mixtures.

EXPERIMENTAL

Throughout the investigation male weanling rats of the Sprague-Dawley strain, 21 days of age and weighing 40 to 45 gm., were employed. Each experiment was set up with four or six rats per lot and was continued for 28 days unless otherwise indicated. The purified basal diet described elsewhere (2) was fed *ad libitum*. All changes in this basal diet were made at the expense of or to the advantage of the dextrin portion.

The amino acid mixture was composed of crystalline amino acids and was essentially that of Rose *et al.* (3), except that the phenylalanine content was increased from 1.20 gm. to 1.60 gm. per 100 gm. of ration. The

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composition of the amino acid mixtures as used is presented in Table I. NaHCO_3 , stoichiometric with the amount of HCl present in the salts of arginine, histidine, and lysine, was added. Proportionate amounts of these basic mixtures were incorporated into the ration. Diammonium citrate (anhydrous) was used as a source of inorganic nitrogen.

For the nitrogen balance studies, animals were placed in individual wire mesh metabolism cages equipped with glass funnels. Urine was collected in bottles containing sulfuric acid and toluene; feces were collected

TABLE I
Composition of Amino Acid Mixtures

	Mixture A	Mixture B	Mixture C
	gm.	gm.	gm.
Valine.....	2.00*	2.00*	1.00
Leucine.....	1.20	1.20	1.20
Isoleucine.....	1.60*	1.60*	0.80
Methionine.....	0.80*	0.80*	0.80*
Threonine.....	1.40*	1.40*	0.70
Phenylalanine.....	1.60*	1.60*	1.60
Lysine-HCl.....	1.50	1.50	1.50
Histidine-HCl.....	0.95	0.95	0.95
Arginine-HCl.....	0.50	0.50	0.50
Tryptophan.....	0.40	0.40	0.40
Glycine.....	0.10		
Alanine.....	0.40*		
Serine.....	0.20*		
Cystine.....	0.20		
Tyrosine.....	0.60		
Proline.....	0.20		
Hydroxyproline.....	0.10		
Aspartic acid.....	0.20		
Glutamic “.....	2.00		

* Racemic.

separately on wire screens. Total nitrogen was determined by the Kjeldahl method. Ammonium nitrogen was determined by the modified (6) aeration method of Van Slyke and Cullen.

The D-amino acid oxidase activity of homogenized liver and kidney from representative animals was determined by the method of Axelrod, Sober, and Elvehjem (7). The substrate was 0.05 M DL-alanine. A quantity of homogenate equivalent to 200 mg. of fresh liver or 100 mg. of fresh kidney was used in each flask. All values recorded are corrected for the endogenous oxygen uptake of an equal quantity of the same homogenate but in the absence of alanine.

Results

The data in Table II demonstrate that the addition of ammonium citrate to diets containing either 4, 8, or 12 per cent of a mixture of ten essential amino acids (Mixture B, Table I) permits greater growth in weanling rats than that which is obtained when no nitrogenous supplement is fed. When the basal ration contained either 4 or 8 per cent of the essential amino acids, the maximum growth response was obtained with 4 per cent of am-

TABLE II
Growth of Rats Fed Diets Containing Amino Acids and Various Supplements

Essential amino acids in ration	No. of animals	Supplement	Nitrogen in ration from supplement	Mean weight gain after 28 days	Mean weight gain after 42 days
<i>per cent</i>			<i>per cent</i>	<i>gm.</i>	<i>gm.</i>
4	8	None		11 ± 1.0*	19 ± 1.7 (4)†
4	4	4% ammonium citrate	0.495	44 ± 2.2	61 ± 4.1 (4)
4	4	6% " "	0.743	35 ± 3.1	
8‡	16	None		33 ± 1.1	47 ± 2.6 (8)
8	4	1.07% ammonium citrate	0.133	42 ± 2.6	
8	8	2.15% " "	0.266	57 ± 2.6	
8	16	4% " "	0.495	76 ± 1.8	133 ± 1.3 (8)
8	4	6% " "	0.743	76 ± 4.8	
8	4	3.3% sodium " "		28 ± 2.0	
8	4	2.6% non-essential amino acids	0.264	54 ± 4.8	
8	4	2.3% urea	1.072	72 ± 5.3	
12	8	None		54 ± 1.2	87 ± 2.3 (4)
12	4	4% ammonium citrate	0.495	75 ± 1.7	138 ± 1.8 (4)

* Probable error of the mean = $k\sqrt{\sum d_a^2/n(n-1)}$ where k = Jensen's constant, and d_a = individual deviations from the arithmetic mean.

† The figures in parentheses refer to the number of animals studied for the extended period.

‡ 8 per cent of amino acids provides 1.014 gm. of N per 100 gm. of ration.

monium citrate. Increasing the amount of ammonium salt to 6 per cent gave no further growth response when 8 per cent of essential amino acids was fed and appeared to depress growth when the diet contained only 4 per cent of essential amino acids. Supplementation with ammonium citrate gave the same growth response as supplementation with a mixture of non-essential amino acids containing the same amount of nitrogen (Mixture A, Table I, substituted for Mixture B). The beneficial effect of ammonium salts is even more apparent after 42 days than at 28 days. These results are in agreement with the findings of Rose *et al.* (5).

Sodium citrate (at a level equivalent to 2.15 per cent ammonium citrate) had no beneficial effect on the growth of rats fed a ration containing 8 per cent of Mixture B. Thus the citrate ion was not responsible for the weight gain. The nitrogen of urea appeared also to be available when limited amounts of essential amino acids were fed.

As shown previously (2) and in Table III, the improved growth rate which results from the addition of ammonium salts to the basal ration is probably due to retention of ammonium nitrogen and its utilization for protein synthesis. Neither fecal nor urinary nitrogen excretion is any greater from rats fed essential amino acids and ammonium salts than from rats fed a mixture of eighteen amino acids. The per cent of dietary N

TABLE III
Nitrogen Balance Studies

	Lot I	Lot II	Lot III	Lot IV	Lot V
	Mixture A*	Mixture B		Mixture C	
(NH ₄) ₂ C ₆ H ₅ O ₇ in ration, %		2.15			2.15
Total N in ration, %	1.26	1.28	1.01	0.82	1.09
Weight gain during 2nd-4th wks., gm.	48, 43	66, 51	24, 27	29, 27	47, 48
Average daily N intake, mg.	130.8	148.2	95.9	66.8	126.8
" " NH ₄ -N intake, mg.		30.8			30.9
" " fecal N, mg.	21.2	17.9	17.3	13.9	16.0
" " urinary N, mg.	30.8	31.4	23.9	10.9	14.1
" " " NH ₄ -N, mg.	1.93	4.0	0.64	0.36	2.13
Dietary N retained, %	60.0	66.5	64.3	63.3	76.5

The values are averages for two rats per lot. Data for each rat were obtained for 6 consecutive days during the 4th and 5th weeks of the experiment.

* Tyrosine was omitted from this ration, thus lowering the proportion of non-essential amino acids to 2.3 per cent.

retained was about the same for each of the five lots. The rats fed ammonium salts (Lots II and V, Table III) excreted urinary ammonium salts equivalent to less than 15 per cent of the amount ingested. These results again are in harmony with the balance studies of Rose *et al.* (5).

A diet containing 6.4 per cent of physiologically active essential amino acids and 2.15 per cent of ammonium citrate (Lot V, Table III) supported as good growth as did a diet containing 10.3 per cent of amino acid Mixture A (Lot I).

In agreement with earlier work (8), supplementation with ammonium salts was found not to increase growth of rats fed natural proteins such as fibrin or soy bean oil meal in limited amounts (4 to 12 per cent of the ration).

Attempted Replacement of Certain Essential Amino Acids—Scul and Rose (9) demonstrated the ability of the rat to synthesize arginine but at a rate insufficient to support maximum growth (10). The possibility remains that the formation of ammonia from other amino acids is the rate-limiting reaction in the synthesis of arginine. Rats, fed approximately 8 per cent of a mixture of the nine absolutely essential amino acids and the quantity of ammonium citrate shown previously to give maximum growth, gained 75 per cent as much weight as those fed the same ration plus 0.33 per cent arginine hydrochloride (Table IV). The respective weight gains of 61 and 83 gm. may indicate that arginine has less effect on growth when

TABLE IV
Attempted Replacement of Essential Amino Acids

No. of animals	Diet	Mean weight gain	
		7 days	28 days
		gm.	gm.
2	10 essential amino acids*	13.5	
2	Less threonine	-20.0	
2	" " + DL- α -aminobutyrate†	-19.5	
2	" " + α -ketobutyrate†	-14.5	
2	" " + DL- α -hydroxybutyrate†	-14.0	
4	10 essential amino acids*	16.5 \pm 0.5	83 \pm 1.9
3	Less arginine	12.3 \pm 0.7	61 \pm 2.9

The rats fed the threonine analogues were 7 weeks old at the start of the experiment and had been fed a diet containing 8 per cent of the essential amino acids plus 4 per cent diammonium citrate for 4 weeks. The experiments involving the possible replacement of arginine dealt with rats of weanling age.

* The diet contained 8 per cent amino acids and 4 per cent diammonium citrate.

† In amounts equivalent to the threonine of Mixture B.

ammonium salts are fed than when non-essential amino acids supplement the nine essential amino acids, for Borman *et al.* (10) obtained a gain of only 39 gm. in 28 days when arginine was omitted and of 72.6 gm. when arginine was fed.

The discovery of Teas, Horowitz, and Fling (11) that homoserine is a common precursor of both methionine and threonine in a mutant of *Neurospora crassa* led us to test the effectiveness of another 4-carbon amino acid DL- α -aminobutyrate, and its α -keto and α -hydroxy analogues as possible substitutes for threonine in diets containing ammonium citrate. None of the three substituted butyric acids was effective in replacing threonine. The negative results with DL- α -aminobutyric acid confirm the recent report of Armstrong and Binkley (12).

Effect of Dietary Nitrogen on D-Amino Acid Oxidase—The D-amino acid oxidase activity of liver and kidney tissue from representative animals was measured (Table V). Feeding ammonium salts or urea with a mixture of essential amino acids was found not to influence the activity and these data are therefore not shown. The concentration of this enzyme in liver increased with increasing intake of protein or amino acids. Kidney D-amino acid oxidase was not influenced by dietary protein level in these experiments. Depriving rats of threonine for 7 days appeared to decrease the D-amino acid oxidase activity of their liver. The decrease was not as striking as that caused by feeding a protein-free diet for 7 days (13).

TABLE V
Effect of Dietary Nitrogen on D-Amino Acid Oxidase

Nitrogen source	No. of animals	Oxygen uptake, μ l. per hr. per mg. N	
		Liver	Kidney
4% fibrin	6	11.8 \pm 0.6*	98 \pm 3.1
8% "	3	17.5 \pm 2.0	97 \pm 10.1
12% "	4	24.1 \pm 0.9	94 \pm 6.6
4% amino acids†	3	11.7 \pm 0.9	96 \pm 17.8
8% " "	5	18.7 \pm 1.9	102 \pm 6.3
8% " " less threonine	7	13.8 \pm 0.6	101 \pm 3.4
19.5% soy bean oil meal (8% protein)	3	17.5 \pm 1.4	138 \pm 5.7

* Probable error of the mean.

† Mixture B.

DISCUSSION

It has been clearly demonstrated, both in this work and in that of Rose *et al.* (5), that supplements of ammonium nitrogen can replace the non-essential amino acids in the diet of growing rats. This in no way implies that the utilization of ammonium nitrogen is limited to its incorporation into the structures of the non-essential amino acids. With the established exceptions of lysine (14) and threonine (15), and the possible exception of valine and arginine,¹ each of the "essential" amino acids can be replaced in the diet of growing rats by its corresponding α -keto analogue. Thus it is the peculiar carbon skeleton of each of these molecules which is the dietary essential (compare also (14)). It seems reasonable to expect that, if suitable quantities of these keto acids were available, a mixture of them, the

¹ Apparently the α -keto analogues of valine and arginine have never been tested as dietary substitutes for their respective amino acids. The α -hydroxy analogue of arginine is slightly active (10).

remaining two to four essential amino acids, and ammonium salts, might be found to support growth of young rats.

SUMMARY

Young rats fed a diet containing the ten essential amino acids effect a net utilization of supplementary ammonium nitrogen, as indicated by improved weight gain and net retention of ammonium nitrogen.

Supplements of ammonium citrate did not improve weight gains by rats fed suboptimal amounts of natural proteins.

The D-amino acid oxidase activity of rat liver was proportional to the level of protein or amino acids in the diets fed. Supplementation of these rations with ammonium salts or urea did not influence the concentration of this enzyme.

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AN EFFECT OF PYRIDOXINE DEPRIVATION ON AMINO ACID METABOLISM IN RATS

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It was reported previously (1) that rats deprived of pyridoxine and maintained on a high protein diet, devoid of fat and carbohydrate, exhibited an abnormal response in the level of blood urea after a test dose of alanine. Subsequently, it was found (2) that pregnant women experiencing severe nausea and vomiting in the first trimester gave, after alanine administration, alterations in blood urea closely resembling those observed in pyridoxine-deprived rats. The abnormal urea curve for the human subjects was altered to normal within a few days after supplying pyridoxine. In the animal studies, the basal diet contained a proportion of protein markedly different from that used commonly by humans. It seemed advisable to reinvestigate amino acid metabolism in rats fed a low protein diet, and to extend the study by measuring alterations in several metabolites, in addition to urea, which might be formed from alanine. This study has been given further interest by the observations of Greenberg *et al.* (3) that vitamin B₆ deprivation in humans causes abnormal metabolism of another amino acid, tryptophan.

Methods

Young white rats of the Wistar strain, with initial weights of 70 to 90 gm., were housed in individual, screen-bottomed cages, and supplied with food and water *ad libitum*. The basal diet contained the following ingredients in per cent by weight: casein 11, sucrose 79, salts mixture (Steenbock-Nelson 40 (4)) 4, agar 2, vitamin powder 4, choline 0.2, inositol 0.2, and cod liver oil concentrate 0.015 (calculated to supply 45 i.u. of vitamin A and 11 i.u. of vitamin D per rat per day). The vitamin powder was prepared by mixing thoroughly 800 gm. of casein with the following amounts (in mg.) of vitamins: thiamine chloride 100, riboflavin 100, calcium pantothenate 400, nicotinic acid 400, *p*-aminobenzoic acid 400, biotin 20, and folic acid 20.

Two analogous studies have been conducted and, since the results have been in agreement, details for one series only will be reported. 100 rats were divided into twelve groups, comparable with regard to distribution

of sex and initial weight. The animals received the basal diet for a period of 15 days. Throughout this time, six groups were given a supplement of 50 γ of pyridoxine hydrochloride per rat per day in the food. On the 14th day, the average weight of the rats receiving pyridoxine was 30 per cent greater than that of the deprived animals. The weight difference was thought to be of sufficient magnitude to indicate a definite effect of pyridoxine insufficiency. The animals were fasted until the morning of the 15th day. Following a fast of 15 hours, animals in one deprived and in one control group each received 5 cc. of 0.9 per cent saline solution intraperitoneally and were sacrificed immediately to obtain blood. Rats in the other ten groups were each given 5 cc. of 7.6 per cent aqueous solution

TABLE I

Amounts of Several Metabolites in, and Packed Cell Volume of, Blood of Rats after Alanine Administration

Hrs. after injection	Urea		Uric acid		Amino nitrogen		Free glutamic acid		Blood sugar		Packed cell volume	
	Control	Deprived	Control	Deprived	Control	Deprived	Control	Deprived	Control	Deprived	Control	Deprived
	Mg. per cent whole blood	Mg. per cent whole blood	Mg. per cent whole blood	Mg. per cent whole blood	Mg. per cent whole blood	Mg. per cent whole blood	Mg. per cent plasma	Mg. per cent plasma	Mg. per cent whole blood	Mg. per cent whole blood		
0	17.4	20.4	1.5	2.5	10.3	10.6	3.8	3.9	92	107	41.5	44.0
1	55.6	47.7	2.1	2.1	34.8	57.0	11.5	14.8	140	110	56.0	55.4
3	92.4	83.4	1.7	2.0	20.8	49.6	3.7	12.2	160	180	53.7	58.7
6	142.8	152.2	1.7	2.4	17.8	24.0	3.4	6.2	142	156	54.0	60.0
9	144.0	173.6	1.2	1.6	12.6	17.5	3.0	4.2	129	160	46.5	52.5
12	132.0	180.4	2.0	2.5	11.8	12.6	4.2	4.7	170	142	45.5	53.0

of DL-alanine intraperitoneally. At periods of 1, 3, 6, 9, and 12 hours after alanine administration, one group of deprived and one group of supplemented rats were sacrificed. In all cases, animals were anesthetized with nembutal, the heart exposed, and all possible blood removed from the heart by means of a hypodermic syringe. Blood from each group was pooled and heparinized. Livers were removed and those from each group pooled for analysis.

In a control series to determine the effects of intraperitoneal administration of fluid and of prolonged fasting on the measured blood constituents, a group of animals similar in weight, sex, and number was treated in the same manner as the experimental rats except that 0.9 per cent saline was given in place of alanine solution.

On the blood pooled for each group, the following analyses were carried

out in duplicate by the procedures stated: uric acid, Block and Geib (5); amino nitrogen, modified procedure of Folin (6); free glutamic acid, Prescott and Waelsch (7); urea, Archibald (8); sugar, Nelson (9). Packed cell volumes for each lot of blood were determined by the standard procedure. Analytical data reported in Table I are averages of the duplicate values found for the pooled blood from each group of animals. Duplicate determinations of total vitamin B₆ were made on livers pooled from each group by the yeast microbiological procedure of Atkin *et al.* (10).

Results

Table I lists the values obtained for urea, amino nitrogen, free glutamic acid, blood sugar, uric acid, and packed cell volumes. The average liver content of total vitamin B₆ was 9.9 γ per gm. of moist tissue for the control groups and 6.1 γ per gm. of moist tissue for the deprived groups.

In the control series the values obtained for urea, amino nitrogen, blood sugar, and free glutamic acid showed no change from their respective fasting levels. These findings eliminated, apparently, the possibility that the effects observed following administration of alanine were due to the injection of considerable liquid or were the result of prolonged fasting.

DISCUSSION

The observed rapid increase in blood amino nitrogen after intraperitoneal administration of alanine would be expected. The rate of subsequent decrease of amino nitrogen and of increase of blood sugar might be indicative of the rate of deamination. The reported data suggest that the rate of deamination was retarded in rats not supplied with pyridoxine.

Assuming that glutamic acid is formed from alanine by transamination, the observed rate of increase in blood glutamic acid after alanine administration could be used as an index of the amount of transamination. However, this interpretation is made difficult by possible alterations in glutamic acid utilization. The reported data suggest that transamination proceeded to at least the same extent in rats receiving pyridoxine as in those not furnished this vitamin. The data indicate, also, that the deprived animals showed a slower utilization of glutamic acid.

The prolonged maintenance of a high level of blood urea after alanine administration has been found to be characteristic of rats not supplied with pyridoxine. This might be caused by abnormal continuation of urea formation or by impaired renal function. Kidney function tests were not carried out, but blood uric acid remained at normal levels in all animals. Changes in blood concentration were not sufficient to account for the observed alterations in blood urea, or indeed in the other metabolites measured.

SUMMARY

Amino acid metabolism in pyridoxine-deprived and in control rats has been studied by the use of a test load of alanine. Pyridoxine insufficiency, to the degree attained in white rats maintained on a pyridoxine-free, low protein diet, appeared to produce no interference with the formation of glutamic acid, urea, or sugar, from alanine. However, a delayed disappearance of these metabolites from the blood was observed. The results suggest that pyridoxine insufficiency did not impair transamination but may have delayed deamination.

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UTILIZATION OF ACETATE BY *TETRAHYMENA GELEII* (S)*

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Tetrahymena geleii (S) (*Colpidium campylum*) is a ciliated protozoan which has been shown to be capable of synthesizing lipides from protein at an unusually high rate (1, 2). The next obvious step in studies concerned with the metabolism of this organism, therefore, seemed to be a search for the compound, or compounds, serving as precursor of the lipide material.

Acetate is known to play a key rôle in the metabolism of lipides, carbohydrates, and proteins (7). Rittenberg and Bloch (8, 9) and Ponticorvo, Rittenberg, and Bloch (10) have shown that acetate may condense to form fatty acids in mammalian tissue. Smedley-MacLean and Hoffert (11) and White and Werkman (12) have demonstrated that in yeast, also, the intact acetate molecule is converted to lipide material. It has also been demonstrated (13-16) that acetate may condense to form higher, non-reducing carbohydrates. The classical work of Schoenheimer and Rittenberg (17) demonstrated that carbohydrate may be transformed into lipides.

It therefore seemed of interest to investigate the fate of acetate utilization by *T. geleii* (S) in an attempt to ascertain whether acetate can serve as a precursor of lipides, and, if so, whether it must first be converted to carbohydrate.

Materials and Methods

Pure, sterile cultures of *T. geleii* (S) were maintained in 2 liter Erlenmeyer flasks containing 1 liter of 1.5 per cent Difco proteose-peptone from which the lipides had been extracted by hot alcohol-ether (18) and the carbohydrates precipitated with copper sulfate (19). New cultures were inoculated with a 25 ml. portion of a 72 hour culture and incubated at 26° for 72 hours. At the end of the incubation period, the organisms

* The strain of *T. geleii* used is the same strain which has been studied as *Colpidium campylum* by the author (1-4) as well as by others (5, 6) at Fordham University during 1948-49. J. O. Corlies (personal communication) recently has established that this organism is *Tetrahymena geleii*. The growth characteristics of this strain differ from those of other strains of *T. geleii* which have been reported in the literature. Since this strain was isolated from a wild culture by the author in 1946, it is a new strain, designated (S).

were concentrated by gentle centrifugation (750 r.p.m.), washed three times with sterile Hahnert's solution (20), and fasted in the third wash for 12 hours. The cells were then again concentrated by centrifugation, given a final washing in Hahnert's solution, and the volume adjusted to yield approximately 10 mg. of cells (dry weight) per ml.

Oxygen uptake was measured by the conventional Warburg direct method. In all cases the total volume of each flask was 3.0 ml. Con-

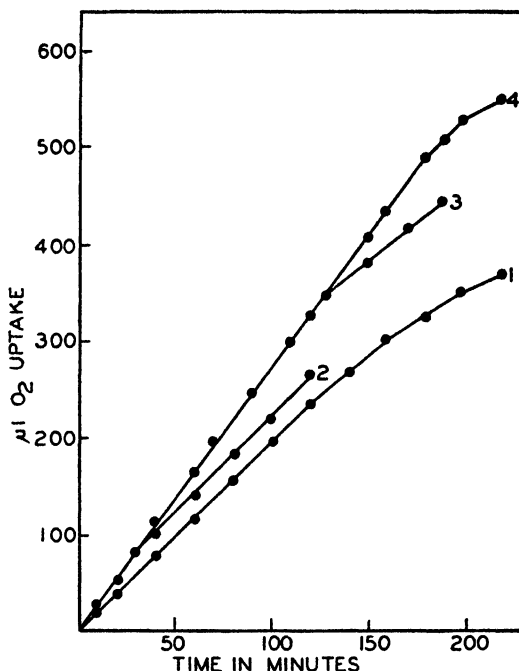


FIG. 1. Effect of acetate on O_2 consumption by *Tetrahymena*. 10.8 mg. (dry weight) of cells; Hahnert's solution, pH 6.7; temperature, 25.6°; gas phase, air. Curve 1, endogenous; Curve 2, 0.001 M acetate; Curve 3, 0.005 M acetate; Curve 4, 0.01 M acetate. Acetate in side arm of flasks and tipped at 0 time.

centrations of substrates and inhibitors are expressed as final concentration.

Acetate was estimated both by steam distillation followed by titration with 0.01 N sodium hydroxide and by the colorimetric method of Hutchens and Kass (21). When small amounts of acetate were expected, the distillation procedure was carried out; when larger amounts ($>80 \gamma$) were anticipated, the more convenient colorimetric method was used. Succinate was estimated by the method of Krebs (22) after ether extraction in a Kutscher-Steudel extractor as modified by Umbreit (23); fumarate

TABLE I

Effect of Acetate on Formation of Carbohydrate and Lipide by T. geleii

Analyses were made on aliquots of the same sample of cells before and after incubation in the presence of 0.05 M acetate. Hahnert's solution, pH 6.7; gas phase, air; temperature, 25.6°.

Dry weight of cells	Additional O ₂ utilized due to acetate	Carbohydrate (as glucose)		Lipide	
		Before incubation	After incubation	Before incubation	After incubation
mg.	μ l.	mg.	mg.	mg.	mg.
7.0	96	0.007	0.019	0.267	0.586
8.4	92	0.008	0.021	0.320	0.685
8.3	93	0.008	0.017	0.319	0.674
6.4	95	0.006	0.020	0.245	0.594
9.2	99	0.009	0.022	0.352	0.723
11.4	91	0.011	0.025	0.425	0.836
10.1	94	0.010	0.024	0.385	0.737

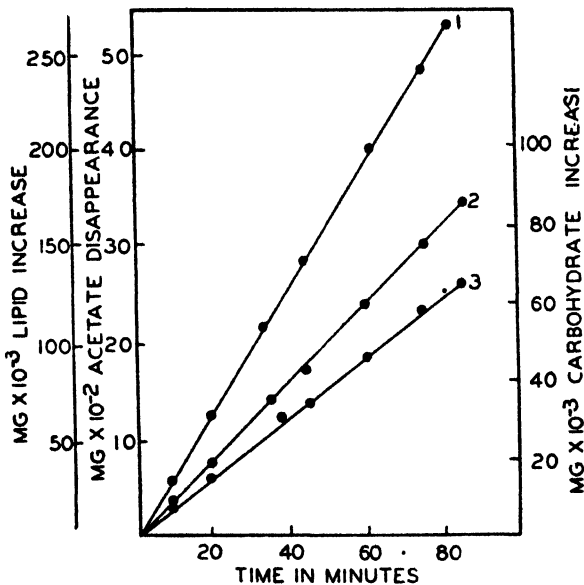


FIG. 2. Rate of lipid and carbohydrate formation from acetate. 9.6 mg. (dry weight) of cells incubated with 0.005 M acetate in Hahnert's solution, pH 6.7; temperature 25.6°; gas phase, air. Curve 1, acetate utilized; Curve 2, lipide synthesized; Curve 3, carbohydrate synthesized.

by the colorimetric method of Marshall, Orten, and Smith (24), and acetyl phosphate by the method of Lipmann and Tuttle (25).

Acetaldehyde was trapped by adding sodium sulfite to the incubation mixture at the beginning of the experiment. At the end of the run, the

mixture was deproteinized with sulfuric acid-sodium tungstate, and the acetaldehyde freed from the filtrate by the addition of dilute HCl and distilled and estimated by the method of Stotz (26). Total carbohydrate was estimated by hydrolyzing an aliquot of a zinc sulfate-barium hydroxide protein-free filtrate with 2 per cent HCl for 3 hours, and estimating the glucose concentration, after neutralization, by the method of Park and Johnson (27).

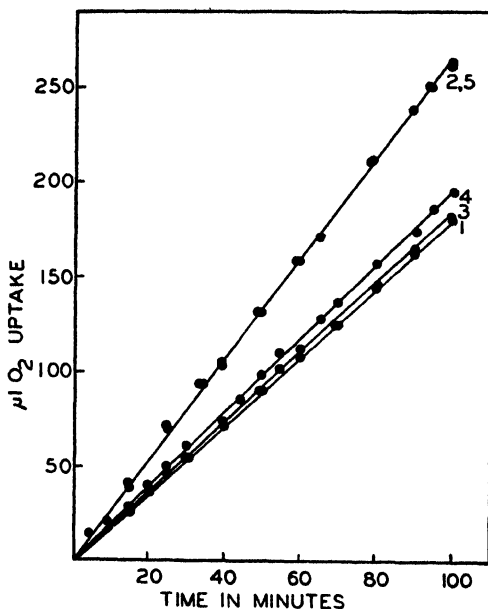


FIG. 3. Effect of fluoride, arsenite, and malonate on acetate oxidation. 9.7 mg. (dry weight) of cells; Hahnert's solution, pH 6.7; temperature, 25.6°; gas phase, air. Curve 1, endogenous; Curve 2, acetate; Curve 3, acetate + 0.02 M arsenite; Curve 4, acetate + 0.01 M malonate; Curve 5, acetate + 0.02 M fluoride. In all cases the acetate concentration was 0.005 M. Inhibitor in main compartment during equilibrium period. Acetate tipped from side arm at 0 time.

Since 98 per cent of the lipides synthesized by *T. geleii* (S) are in the form of fatty acids (2), lipid determinations were made with the micro-method of Jones (28) after saponification with sodium hydroxide.

Results

The effect of various concentrations of sodium acetate on the oxygen consumption of *T. geleii* (S) is shown in Fig. 1. Added acetate at a concentration of 0.01 M results in an additional oxygen uptake of 17 μ l., of 97 μ l. at 0.005 M concentrations, and of 196 μ l. at a concentration of 0.01 M. These values represent only 14 per cent complete oxidation of the

utilized acetate. Estimations of acetate at the time of the "break" in the respective curves indicate that at this point all of the added acetate had been utilized; none could be recovered.

The amounts of lipide and carbohydrate recovered in the presence and in the absence of added acetate are shown in Table I. In the presence of acetate there is an increase in fatty acids of 91 to 145 per cent (mean, 112 per cent) and a 113 to 233 per cent (mean, 156 per cent) increase in carbohydrate. Fig. 2 shows that there is a constant, straight line increase in amounts of both lipide material and carbohydrate as acetate is utilized by *Tetrahymena*.

The effect of the inhibitors, sodium fluoride, arsenite, and malonate, on the oxidation of acetate is shown in Fig. 3 and their effect on the formation of carbohydrate and lipide in Table II. Fluoride, as would be ex-

TABLE II

Effect of Arsenite, Malonate, and Fluoride on Formation of Carbohydrate and Lipide by T. geleii in Presence of Added Acetate

7.2 mg. (dry weight) of cells; incubation period, 175 minutes; Hahnert's solution, pH 6.7; gas phase, air; temperature, 25.6°. Concentration of acetate, 0.005 M; arsenite, 0.02 M; malonate, 0.01 M; fluoride, 0.02 M.

Flask additions	Carbohydrate (as glucose) recovered	Lipide recovered
	mg.	mg.
Acetate	0.007	0.264
" + arsenite	0.021	0.627
" + malonate	0.020	0.605
" + fluoride	0.021	0.618
" + fluoride	0.006	0.038

pected, does not inhibit the oxidation of acetate. Rather, its effect is to inhibit completely the synthesis of carbohydrate, probably by blocking essential phosphorylative steps. Arsenite and malonate, on the other hand, have their effect in inhibiting the oxidative pathway and have no significant effect on the rate of lipide and carbohydrate formation from acetate. Arsenite inhibits oxygen uptake, due to added acetate, by 96 per cent; malonate by 86 per cent.

The inhibition of oxidation of acetate in the presence of malonate and arsenite should result in the accumulation of succinic and fumaric acids, due to blockage at the level of succinic dehydrogenase and fumarase respectively. Table III shows the amounts of succinic and fumaric acids recovered when acetate is incubated in the presence of malonate and in the presence of arsenite. In the absence of the inhibitors, the amounts of succinic and fumaric acids were too small to be detected with the methods used.

In an attempt to obtain an indication as to the "active" 2-carbon compound in the condensation of acetate to lipide, cells were incubated with acetate and sulfite to trap acetaldehyde. Fluoride was used to aid in the accumulation of acetyl phosphate. Table IV shows the amounts

TABLE III

Recovery of Succinate and Fumarate from T. geleii Incubated with Malonate or Arsenite

Succinate determinations carried out on cells incubated in 0.005 M acetate in presence of 0.01 M malonate; fumarate determinations on cells incubated in 0.005 M acetate in presence of 0.02 M arsenite. Incubations for both determinations were run simultaneously on aliquots of the same sample of cells. Incubation time, 90 minutes; gas phase, air; temperature, 25.6°.

Dry weight of cells	Incubation in acetate + malonate		Incubation in acetate + arsenite	
	Acetate disappearance	Succinate recovery	Acetate disappearance	Fumarate recovery
mg.	mg.	mg.	mg.	mg.
6.9	0.436	0.029	0.417	0.014
7.9	0.516	0.033	0.471	0.015
9.8	0.649	0.041	0.536	0.022
9.4	0.640	0.039	0.559	0.017
10.1	0.669	0.042	0.600	0.019

TABLE IV

Recovery of Acetaldehyde from T. geleii Incubated with Acetate and Sulfite

Hahnert's solution, pH 6.7. Each flask contained, in addition to cells in buffer, 0.005 M acetate and 0.01 M Na_2SO_3 . Temperature, 25.6°; gas phase, air; incubation period, 60 minutes.

Dry weight of cells	Acetate disappearance	Acetaldehyde recovered
mg.	mg.	mg.
9.6	0.206	0.091
7.4	0.161	0.070
8.2	0.189	0.083
10.1	0.218	0.092
9.4	0.209	0.089
9.2	0.211	0.094
6.8	0.147	0.059

of acetaldehyde recovered when acetate is incubated with sulfite. All attempts to recover acetyl phosphate met with negative success.

DISCUSSION

Since the rates of formation of carbohydrate and of lipides are constant during the utilization of acetate (Fig. 2), it appears that both lipides and

carbohydrates are synthesized directly from acetate, the carbohydrate phase not being essential as a precursor of lipide. Additional evidence for this view is the fact that fluoride, which inhibits completely the synthesis of carbohydrate, has no effect on the amount of fatty acids formed from acetate (Table II). These results are, in general, in accord with those obtained with yeast by MacLeod and Smedley-MacLearr (29). The mode of utilization of acetate by *T. geleii* (S) is thus not similar to that which occurs in such forms as *Prototheca zopfii* (30) and *Pseudomonas calcoacetica* (31) in that in *Tetrahymena* the main products of acetate assimilation are both fat and carbohydrate, rather than carbohydrate alone.

Stadtman and Barker (32) have shown that the synthesis of fatty acids by *Clostridium kluyveri* proceeds by way of acetaldehyde oxidation to acetyl phosphate which in turn condenses with acetate, forming butyrate. The occurrence of such a mechanism in *Tetrahymena*, indeed of any scheme of lipide formation from acetate by way of acetyl phosphate, cannot be considered at this time, in view of the observation that fluoride has no inhibitory effect on the amount of lipides formed, as well as the fact that all attempts to recover acetyl phosphate were negative. The occurrence of acetaldehyde as an intermediate in the production of fat from acetate by yeast has been demonstrated by Smedley-MacLean and Hoffert (33). White (34) also recovered acetaldehyde during the synthesis of fat from acetate by yeast, and, as was the case in the present investigation, was unable to demonstrate the presence of acetyl phosphate during the course of synthesis.

SUMMARY

Tetrahymena geleii (S) synthesizes carbohydrate and fatty acids simultaneously from acetate. Carbohydrate is not a necessary precursor of lipides during the synthesis.

Fluoride inhibits the formation of carbohydrate from acetate, but has no effect on the amounts of lipides formed. Arsenite and malonate inhibit the oxidation of acetate and have no effect on lipide or carbohydrate recovery.

Succinate and fumarate are recovered when acetate oxidation is blocked with malonate and arsenite respectively.

Acetaldehyde, rather than acetyl phosphate, is probably the "active" 2-carbon compound in the formation of lipides from acetate by *Tetrahymena*.

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EFFECT OF ASCORBIC ACID DEFICIENCY ON COLLAGEN CONTENT OF GUINEA PIG TISSUES*

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In their treatise on human scurvy (1), Aschoff and Koch suggested that the primary abnormality of this disease was the inability of the organism to produce intercellular substances. Wolbach and his collaborators (2-4) came to the same conclusion as the result of experiments performed in guinea pigs. Although they stated (5) that the scorbutic state was characterized by the inability of the guinea pigs to form and maintain intercellular materials, the evidence dealt mostly with the relation of vitamin C to the formation of intercellular substances. Many investigators have directed their attention to this aspect of connective tissue physiology and the experimental evidence is conclusive that, in the absence of ascorbic acid, there is a marked deficiency in the formation of connective tissue and its components.

Little has been written as to the fate of the preformed connective tissue in the vitamin C-deficient state. Höjer (6) wrote that in the guinea pig "there is general atrophy in scurvy of the connective tissue, and especially of its collagen substance." Aschoff and Koch described an inability to maintain established supporting structures in the human. When scurvy supervened on a recently healed wound, the newly formed collagen of the scar reverted to "precollagen;" yet the fibrous tissue bordering the incision remained normal (7).

Controversy still exists concerning the manner in which connective tissue is maintained in the body. Two main theories have been proposed: One view states that there is a dynamic equilibrium in which formation and destruction of the connective tissue occur simultaneously and continuously. The other theory maintains that there is constant anabolism of connective tissue unaccompanied by any appreciable breakdown. It is generally agreed that, in the absence of ascorbic acid, the anabolic processes are inhibited. Whether catabolism proceeds at its normal pace or whether it is accelerated in the absence of vitamin C has not been established. Ham and Elliott (8) proposed that in scurvy there was failure to

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form new connective tissue, rather than acceleration of the destructive phase. In order to establish clearly that vitamin C is essential for the maintenance of preformed collagen, it must be demonstrated that in its absence catabolism of the collagen is accelerated.

A chemical method is available for the quantitative estimation of collagen in tissues (9). It has been used to measure the collagen content of liver (9-14), heart and muscle (9, 15-18), aorta (19), kidney (9, 12), and spleen (9) of different species. This study was undertaken to establish the fate of the preformed collagen in the tissues of guinea pigs placed on an ascorbic acid-deficient diet.

Materials and Methods

Thirty male, weanling guinea pigs were used as experimental animals. They were divided equally into the following groups.

Group 1—These animals received a diet consisting of ground Derwood rabbit chow that had been exposed to the air for at least 24 hours, supplemented with 5 per cent ground dried yeast. Weekly, 0.2 cc. of wheat germ oil and 0.8 cc. of cod liver oil were fed orally. Water was given *ad libitum*. At the start of the experiment these animals weighed between 160 and 200 gm., with a mean weight of 185 gm. They were maintained on this diet for 24 to 38 days, at the end of which time they were sacrificed.

Group 2. Age Controls for Group 1—These guinea pigs received the same diet as Group 1, and, in addition, vitamin C was administered in the form of fresh greens daily. They were of equal body weight to Group 1 at the start of the experiment and were kept on the diet for 24 to 38 days.

Group 3. Weight Controls for Group 1—These animals received the same diet as Group 2 and were selected so that their body weights at the termination of the experiment were the same as those of Group 1.

The animals were sacrificed with a blow to the back of the skull. Their bodies were weighed and dissected and the organs were removed in the following manner and order.

Skeletal Muscle—A sample of approximately 2 gm. of adductor muscle of the leg was freed of fat, fascia, and tendon. In the animals in which the muscle of one leg was not sufficient, an additional sample was taken from the opposite side.

Heart—The heart was removed from the body and the pericardium was stripped away. The great vessels were severed at their bases. The cardiac chambers were opened and the blood was removed.

Lungs—The lungs were divested of the mediastinal structures by severance at their hila. The pulmonary vessels were allowed to drain free of blood.

Liver—The diaphragm, portal structures, and gallbladder were separated

from the liver; the hepatic blood was drained and the remaining organ was weighed to the nearest 0.1 gm.

Kidneys—The capsules were stripped away from the surface of the kidneys and the renal vessels and ureters were severed at the uretero-pelvic junction. The kidneys were sectioned longitudinally and the blood was wiped away.

Spleen—The spleen was removed, free of its vessels, fat, and mesentery.

Quantitative chemical determinations of the collagen content of the various tissues were made (9). In each instance the entire organ was used for the measurement, except for the skeletal muscle and liver of which 2 to 4 gm. of samples were used. The tissues were utilized within 1 hour.

TABLE I
Mean Collagen Content of Lungs and Liver

Group No. (10 guinea pigs each)	Body weight	Lungs			Liver		
		Weight	Collagen, wet weight	Total collagen	Weight	Collagen, wet weight	Total collagen
	gm.*	gm*	per cent*	mg.*	gm.*	per cent*	mg.*
1. Scorbatic	176	1.66	1.14	18.9	10.1	0.32	32.3
	±13	±0.10	±0.05	±0.9	±0.8	±0.07	±6.6
2. Age controls	358	2.13	1.55	33.0	17.0	0.66	112.2
	±14†	±0.07†	±0.09†	±2.0†	±0.8†	±0.11	±18.6†
3. Weight controls	173	1.51	1.06	16.0	7.6	0.43	32.7
	±11	±0.09	±0.13	±2.5	±0.9	±0.08	±9.6

* Includes standard error of the mean.

† Indicates significant difference ($P \leq 0.01$) from the mean of the scorbatic group.

after the animals were sacrificed. Rarely, the tissues were stored in sealed containers for no longer than 24 hours at 0° prior to use.

Results

Progress of Vitamin C Deficiency—The animals on the scorbutogenic diet (Group 1) gained weight during the first 2 weeks of the experiment and appeared healthy and active. In the 3rd and successive weeks, their weights became stabilized and then declined, so that at the termination of the experiment the mean weight of this group was 176 gm., with a range of 130 to 255 gm. The animals were listless and apathetic; gingival hemorrhages, subcutaneous ecchymoses, and swelling and reddening of the joints of the extremities were present. Examination of the tissues revealed hemorrhages in the skeletal muscles and periarticular soft tissues. The animals of Group 2 gained weight normally and appeared healthy. They

were sacrificed at the same time as the scorbutic animals and weighed between 300 and 440 gm., with a mean of 358 gm. They displayed none of the stigmata of the scorbutic state. The animals of Group 3 were selected from a normal population so that they were equivalent in weight to the

TABLE II
Mean Collagen Content of Kidneys and Spleen

Group No. (10 guinea pigs each)	Body weight	Kidneys			Spleen		
		Weight	Collagen, wet weight	Total collagen	Weight	Collagen, wet weight	Total collagen
		gm.*	gm.*	per cent*	gm.*	per cent*	mg.*
1. Scorbutic	176	2.81	0.41	11.5	0.44	0.71	3.12
	±13	±0.15	±0.03	±0.8	±0.05	±0.18	±1.00
2. Age controls	358	3.18	0.66	21.0	0.48	0.73	3.50
	±14†	±0.15	±0.07†	±3.1†	±0.03	±0.11	±0.57
3. Weight controls	173	1.98	0.41	8.2	0.27	0.67	1.81
	±11	±0.11†	±0.02	±0.9	±0.03	±0.13	±0.31

* Includes standard error of the mean.

† Indicates significant difference ($P \leq 0.01$) from the mean of the scorbutic group.

TABLE III
Mean Collagen Content of Heart and Skeletal Muscle

Group No. (10 guinea pigs each)	Body weight	Heart			Skeletal muscle
		Weight	Collagen, wet weight	Total collagen	Collagen, wet weight
		gm.*	gm.*	per cent*	per cent*
1. Scorbutic	176	0.61	1.39	8.48	1.87
	±13	±0.04	±0.17	±0.40	±0.23
2. Age controls	358	1.00	1.05	10.50	0.91
	±14†	±0.04†	±0.06	±0.90	±0.21†
3. Weight controls	173	0.55	0.82	4.51	0.81
	±11	±0.03	±0.07†	±0.51†	±0.06†

* Includes standard error of the mean.

† Indicates significant difference ($P \leq 0.01$) from the mean of the scorbutic group.

scorbutic animals. The mean weight of this group was 173 gm., with a range of 120 to 230 gm. The animals appeared normal in all respects.

Chemical Data—The data giving the weights of the various organs, their collagen content, and the total amount of collagen in each organ are listed in Tables I to III.

Lungs—The lungs of the ascorbic acid-deficient animals (Group 1) were

significantly smaller and contained less collagen than the lungs of the age controls (Group 2). However, no significant differences of these values from those of the weight controls (Group 3) were observed.

Liver—Significant diminution of the weight and total collagen content of the livers of the deficient animals from that of the age controls was recorded. These values were not different from those of the weight-controls.

Kidneys—The kidneys of the scorbutic animals were of approximately the same weight as the comparable organs of their age controls, but were heavier than the kidneys of the weight controls. However, the mean percentage and total collagen content of the kidneys of Group 1 resembled the values of the weight controls and were significantly less than those of the age controls.

Spleen—Very small amounts of collagen (not greater than 3.5 mg.) were present in the spleens of the different groups. No significant differences between the groups could be distinguished.

Heart—The hearts of the scorbutic animals were similar in weight to those of the weight controls and were less than the age controls. The percentage and total collagen content of the hearts resembled those of age controls and were significantly greater than in the weight controls.

Skeletal Muscle—There was marked loss of muscle tissue in the scorbutic animals. The relative amount of collagen (per cent) was markedly increased in the experimental group over either of the control groups.

DISCUSSION

In the development of the ascorbic acid-deficient state the guinea pigs underwent marked changes of nutrition. The appetite of the animals diminished sharply and the weight loss that ensued was severe. During the course of these experiments, the animals of Group 1 at first gained and then lost weight, and at the termination of the experiment they were of the same weight approximately as at the start, 3 to 5 weeks previously. It has been shown that organ weights increase with age in the guinea pig (20) and that variations of the collagen content of these organs are to be expected at different age and weight levels.¹ Therefore, in the interpretation of the results obtained from scorbutic animals, the effects of weight loss and vitamin deficiency alone had to be distinguished. The proper selection of control groups helped to eliminate this factor. By the selection of animals of equal age which were allowed to develop normally, a comparison could be made in which both weight loss and vitamin deficiency played a part. The second group of controls, normal animals of

¹ Elster, S. K., and Lowry, E. L., to be published.

the same weight, allowed a comparison in which vitamin deficiency alone played the major rôle. This technique has been utilized previously in the study of capillary permeability in scurvy (21).

In none of the tissues examined was less collagen found in the scorbutic animals than in both of the control groups. For the most part, the organs resembled in weight and collagen composition the corresponding organs of the weight controls and were less than the age controls. Some exceptions may be cited in that the kidneys grew normally, whereas the other organs remained small. No explanation for this phenomenon is offered. In the cardiac and skeletal muscular tissues, the relative amount of collagen was increased. Although it was not possible to weigh the entire muscle mass of the body, it was obvious that the bulk of the weight loss in the animals was due to the loss of muscle substance, a condition that was described previously (22). The relative increase of the connective tissue was due to accretion rather than to an increase of the collagen. The collagen content of the heart of the scorbutic animals resembled that of the age controls rather than of the weight controls. Since the heart specimens contained valves, chordae tendineae, and auricles, structures composed of large amounts of dense, compact collagen, one may think of these sites as containing relatively "stable" collagen. This is in contrast to the state of the connective tissue of the other organs in which the collagen is deposited in more delicate fibers and is, therefore, more susceptible to resorption.

From these studies, it can be seen that in no organ has there been greater loss of collagen than of other tissue constituents during the course of ascorbic acid deficiency. In muscular tissue, rather, the collagen was less sensitive to destruction than the muscle itself. That ascorbic acid is essential for the formation of collagen *in vivo* cannot be disputed. Once formed, the fiber seems to be independent of further vitamin C nutrition, except in so far as it is destroyed by the usual catabolic actions of the body and requires resynthesis. In the true sense, therefore, ascorbic acid may not be essential for the maintenance of preformed connective tissue.

The rôle of ascorbic acid in collagen formation has not been well elucidated. Meyer (23) has suggested that vitamin C may be incorporated into chondroitinsulfuric acid which may then be used in collagen synthesis. Penney and Balfour (24) have shown that hyaluronic acid, a component of connective tissue ground substance, was not formed in the scorbutic state in the guinea pig. Ascorbic acid has been demonstrated to decrease the viscosity of collagen *in vitro* in the presence of hydrogen peroxide (25). Studies (26, 27) have yielded conflicting results as to the rôle of vitamin C in fiber formation *in vitro*. The adrenal cortical hormones have become of increasing interest and importance in connective tissue physiology. Ragan and his collaborators (28) have recently demonstrated that corti-

sone inhibited the formation of granulation tissue, a condition also seen in the ascorbic acid-deficient state. The relation between these two facts is a fertile field for further investigation.

SUMMARY

Male, weanling, guinea pigs were made scorbutic and chemical measurements of the collagen content of the lungs, liver, kidneys, spleen, heart, and skeletal muscles were made. Comparable determinations were performed in normal age and weight controls.

There was a significant decrease of the collagen content of the lungs, liver, and kidneys as compared with age controls, but no significant difference from normal animals of the same weight. A relative (per cent) increase of the collagen content of the heart and skeletal muscle was noted. The total collagen content of the heart was not significantly different from that of the age controls, but was greater than in the weight controls. The collagen content of the spleen was unaffected.

In no instance was less collagen found in the tissues of scorbutic guinea pigs than in normal animals of the same weight.

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THE SULFUR BALANCE OF RATS FED EXCESS DL-METHIONINE PLUS GLYCINE OR DL-ALANINE*

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It has been demonstrated that when mature, male, Sherman strain rats are fed 4.8 per cent DL-methionine while on a 12 per cent casein diet the animals soon attain nitrogen equilibrium or a small positive nitrogen balance (1, 2). The rats lose weight, however, even in positive balance, this loss resulting from a decrease in the fat stores of the body. At the end of 20 days the fat deposits are markedly reduced. A hypertrophy of the kidney of approximately 30 per cent over control values is also noted.

The addition of 4.8 per cent glycine to this diet partly counteracts the loss in weight and kidney hypertrophy. It has been suggested (2) that the glycine may serve as an additional source of the serine (3, 4) needed for methionine catabolism. If this is so, the effect of the glycine should be fairly specific. To test this point extra DL-alanine was fed, and to study further the relationships between the metabolism of fat and methionine and the effects of added glycine, sulfur balances and urinary distribution of sulfur were determined in four groups of rats.

EXPERIMENTAL

Four groups of male, Sherman strain rats, weighing approximately 250 gm. each, were utilized. There were ten rats in each group. The basal diet contained, in addition to the ingredients previously described (2), 1 gm. of choline chloride per 1000 gm. of dry material.

Group I received the basic diet alone, while the other three groups received, in addition to the basic diet, the following ingredients per 1000 gm. of dry material: Group II, 48 gm. (4.8 per cent) of DL-methionine; Group III, 48 gm. (4.8 per cent) of DL-methionine plus 48 gm. (4.8 per cent) of glycine; Group IV, 48 gm. (4.8 per cent) of DL-methionine plus 50 gm. (5.0 per cent) of DL-alanine.

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All of the animals were fed the same weight of diet as was consumed by Group II. Since the animals in this group restrict their intake, the caloric and protein intakes of the other groups are similarly limited. The rats were housed in metabolism cages, two in a cage, and urine and fecal collections made daily for the last 16 days of a 20 day experimental period. The urine samples were preserved with dilute hydrochloric acid and toluene until analyzed. The fecal samples were homogenized with water and an aliquot taken for nitrogen analysis. The remainder was preserved with concentrated nitric acid for sulfur determinations.

Dietary sulfur was determined by analysis of the diets and expressed in terms of gm. of sulfur per gm. of diet. This, multiplied by the food intake per day, gave the daily sulfur ingestion. The sulfur contents of the

TABLE I

Effect of Dietary Supplements of DL-Methionine, Glycine, and DL-Alanine on Nitrogen Balance and Body Weight of Rats during 20 Days

Average of twenty values obtained on ten rats in each group.

Group No.	Supplement			Ingested N	Urinary N	Fecal N	Nitrogen balance	Weight change
	Methionine	Glycine	Alanine					
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>mg. per kg. per day</i>	<i>mg. per kg. per day</i>	<i>mg. per kg. per day</i>	<i>mg. per kg. per day</i>	<i>gm.</i>
I	0.0	0.0	0.0	431	414	85.6	-69	-39.5
II	4.8	0.0	0.0	558	507	78.5	-27	-45.6
III	4.8	4.8	0.0	770	663	78.7	+28	-31.9
IV	4.8	0.0	5.0	755	681	84.6	-11	-45.8

diets, determined by analysis, were found to agree with those values calculated from the sulfur contents of the protein, methionine, and salts.

The urine and fecal samples from each cage were divided into four consecutive 4-day periods, and analysis of each sample performed in duplicate. Pseudocholinesterase was determined by the method of Ammon (5), as modified by Mendel and Mundell (6). Liver fat was determined by the method of Hsiao (7) on the dried livers.

Results

The nitrogen balances for the four groups and the weight losses over the 20 day period are listed in Table I.

All of the groups lost considerable weight in this experiment, but Group III lost 30 per cent less weight than the others.

That the effect of the glycine is not due to the extra nitrogen it affords becomes apparent from a consideration of the data in Table I. The rats in Group IV, fed excess methionine plus alanine, with approximately the

same nitrogen intake as Group III (methionine plus glycine), still lost as much weight as the group fed excess methionine alone (Group II).

The ingestion of 200 mg. per kilo of extra nitrogen in the form of DL-alanine had no significant effect on the nitrogen balance. The ingestion of approximately the same amount of nitrogen as glycine, however, reduced the weight loss and favored a more positive nitrogen balance.

Further confirmation that weight loss takes place largely in fat stores is given by comparison of Group I (casein alone) with Groups II and IV (excess methionine and excess methionine plus alanine respectively). The latter two have less negative nitrogen balances than Group I, but lost more weight, the extra weight representing fat.

Values for plasma pseudocholinesterase, the liver and kidney weights, and liver fat for the four groups are recorded in Table II.

TABLE II

Plasma Pseudocholinesterase Values, Liver Fat, and Liver and Kidney Weights of Rats Fed Dietary Supplements of DL-Methionine, Glycine, and DL-Alanine

. Average of ten values in each group.

Group No.	Supplement			Plasma pseudocholinesterase	Fresh liver weight	Fresh kidney weight	Fat in dried livers	Liver fat
	Methionine	Glycine	Alanine					
	per cent	per cent	per cent	$\mu\text{l. CO}_2$ per hr. per ml. plasma	gm. per 100 gm. body weight	gm. per 100 gm. body weight	per cent	mg. per gm. body weight
I	0.0	0.0	0.0	67.1	2.70	0.578	15.9	1.42
II	4.8	0.0	0.0	35.6	3.38	0.909	20.0	2.01
III	4.8	4.8	0.0	42.6	3.10	0.746	19.8	1.64
IV	4.8	0.0	5.0	64.8	3.22	0.860	26.4	2.71

The effect of glycine in reducing kidney hypertrophy caused by excess methionine is illustrated again in Table II. Alanine reduced kidney hypertrophy only slightly.

There is no correlation of values for pseudocholinesterase with those for liver fat or liver and kidney weights. The functions of this enzyme, therefore, remain obscure.

Sulfur Balance

Fecal and urinary sulfur was determined essentially as described by Eckert (8), with methods by which 99 per cent of added methionine sulfur is recovered. The sulfur balances for the four groups are recorded in Table III.

Consideration of the data in Table III shows excellent absorption of methionine, even at the high levels fed. Groups II and IV have very similar sulfur balances, which again point to the lack of action of alanine.

The addition of the extra glycine, however, increases the total urinary sulfur in comparison to Groups II and IV.

Interestingly enough, Groups II and IV, while both in positive sulfur balance, are in slight negative nitrogen balance. The converse is true of Group III (methionine and glycine) in which the sulfur balance is near equilibrium and yet the nitrogen balance is increased to a positive value.

In order to determine the distribution of the urinary sulfur, sulfur partition was performed on the urine samples. The results are presented also in Table III.

The excretion of inorganic and organic sulfur in Group III (methionine plus glycine) is increased over that of Group II (methionine), even though the latter group ingested more sulfur. The addition of extra alanine to

TABLE III

Effect of Dietary Supplements of DL-Methionine, Glycine, and DL-Alanine on Sulfur Balance and Urinary Sulfur Partition of Rats during 20 Days

Average of twenty values obtained on ten rats in each group.

Group No.	Supplement			Ingested S	Urinary S	Fecal S	Sulfur balance	Organic S*	Total inorganic S
	Methionine	Glycine	Ala-nine						
	per cent	per cent	per cent	mg. per kg. per day	mg. per kg. per day	mg. per kg. per day	mg. per kg. per day	mg. per kg. per day	mg. per kg. per day
I	0.0	0.0	0.0	36.5	33.7	22.5	-20	9.6	24.1
II	4.8	0.0	0.0	313.0	246.0	29.1	+38	87.0	159.0
III	4.8	4.8	0.0	302.0	270.0	31.5	+0.5	100.0	170.0
IV	4.8	0.0	5.0	294.6	235.0	29.6	+30	78.5	156.5

* Ethereal sulfur was negligible in all four groups.

excess methionine actually depresses somewhat the sulfur excretion, although this cannot be considered significant, since the sulfur intake of Group IV was slightly less than that of Group II.

DISCUSSION

When excess DL-methionine (up to 7 per cent of the diet) is fed to Sherman strain rats, practically all of the methionine is absorbed from the gut. The methionine absorbed must either be metabolized, stored, excreted in the urine, or used in the synthesis of protein. The last possibility appears quite unlikely, since it has been demonstrated (9) that the essential amino acids are not utilized for protein synthesis unless all of them are presented to the tissues simultaneously and in proper quantities.

Thus far, the storage of single amino acids has not been demonstrated, although in this work rats fed excess methionine (Group II) or excess methionine plus alanine (Group IV) are in positive sulfur balance and

therefore must be storing sulfur, in some form, to the extent of the respective positive balances. The amount of sulfur stored represents approximately 10 per cent of the ingested sulfur in these two groups. The remaining 90 per cent, and, in the case of Group III (methionine plus glycine) nearly 100 per cent, must be metabolized or eliminated unchanged.

A consideration of the urinary sulfur partition of the control group-fed casein alone (Group I) shows that approximately two-thirds of the eliminated sulfur is oxidized to sulfate, while the other one-third is excreted in organic form. This same proportion is maintained in Group II (methionine) and Group IV (methionine plus alanine), indicating that no single mechanism for sulfur metabolism or elimination is favored, but that all mechanisms for these processes are increased concurrently and by the same amounts. In Group III (methionine plus glycine) approximately 10 per cent more sulfur is excreted, most of the increase being in the organic sulfur fraction. It would seem, therefore, that the glycine facilitates, somewhat, the excretion of the extra sulfur of added methionine, particularly in the organic form.

It has been suggested previously (2) that the beneficial effects of glycine may be due to its direct participation in the catabolism of methionine, since it has been shown (3) that glycine may be the precursor of serine *in vivo* and it has been demonstrated (10-12) that one pathway for the metabolism of methionine is by combination of homocysteine and serine to form cystathionine, which then is cleaved to cysteine and a 4-carbon fragment. When methionine metabolism is channeled, to a greater extent, in this way through organic derivatives, with their subsequent excretion in the urine, this may cause less of a drain on the fat reserves than does complete oxidation to sulfate.

It should be emphasized that methionine fed at high levels does not necessarily exert a marked lipotropic action on liver fat. Best and Ridout (13) have reported that feeding levels greater than 0.5 per cent methionine did not produce a correspondingly greater decrease in liver fat, and they still found a considerable amount in the liver (10 to 17 per cent). This has been confirmed by Channon *et al.* (14).

The liver fat of the animals in Group IV (methionine plus alanine) was higher than in the other groups; three of the animals had fatty livers with fat content up to 38 per cent on a dry weight basis. It is possible that excess alanine favors the oxidation of methyl groups made available by demethylation of methionine in the liver. The net effect would be similar to that obtained by feeding extra cystine or cysteine, which have been shown to cause a large increase in liver fat of rats fed on a low casein-high fat diet (15, 16). Homocysteine also was reported to act in the same manner as cystine on liver fat (17).

At the present time a point has been reached where a more critical evaluation of some of these factors can be made.

SUMMARY

1. Nitrogen and sulfur balances have been determined on four groups of Sherman strain rats fed 4.8 per cent DL-methionine, 4.8 per cent DL-methionine plus 4.8 per cent glycine, and 4.8 per cent DL-methionine plus 5 per cent DL-alanine in addition to a 12 per cent casein diet. Liver fat and plasma pseudocholinesterase values have been reported for the four groups.

2. On a control 12 per cent casein diet, two-thirds of the urinary sulfur excretion is as sulfate and one-third as organic sulfur. This proportion is maintained in the groups fed excess methionine or excess methionine plus alanine.

3. Rats fed excess methionine plus glycine excrete 10 per cent more sulfur than those fed the same quantity of excess methionine alone.

4. A storage of 10 per cent of the ingested sulfur is indicated in the groups fed excess methionine or excess methionine plus alanine.

5. High liver fat is observed in rats fed excess methionine plus alanine.

6. An explanation for these results is advanced.

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QUANTITATIVE FLUOROMETRIC METHOD FOR THE DETERMINATION OF THE NATURAL ESTROGENS*

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This communication deals principally with the development of a method for the quantitative fluorometric measurement of the natural estrogens and introduces data on a similar test method for the quantitative determination of cholesterol. To achieve this goal, use has been made of the condensation reaction of phenolic steroids with phthalic anhydride (1, 2). In view of the wide-spread interest in assay methods for the quantitative determination of urinary androgens, a study was also made of the fluorescence of the reaction products of certain neutral steroids with phthalic anhydride.

Synthesis of Phthaleins of Naturally Occurring Estrogens

Experimental Procedure—Preliminary experiments established the fact that with zinc chloride as the catalyst one could bring about a condensation of each of the three natural estrogens with phthalic anhydride. It remained then to determine the effect of known variables on the reaction *per se* and on the fluorescence of the reaction products. With reference to the condensation reaction, the variable factors studied were moisture, acidity, and the temperature and duration of heating. As for the fluorescence of the solution of the reaction products (phthaleins), the variables investigated were moisture, acidity, type of solvent, optical filters, time of standing after dilution, and light (daylight, artificial light, and ultraviolet).

After establishing the conditions under which the condensation reaction was reproducible quantitatively, standardization curves of the three estrogens were determined by setting up quadruplicate samples of the reagent blank and of each of five concentrations of estrogen, as indicated below.

The detailed experimental procedure is as follows: Experimental samples consisting of 1 ml. or less of a solution of a crystalline estrogen in 95 per cent ethyl alcohol are pipetted into each of four Pyrex test-tubes for each concentration used. To each of these, and to four tubes for the reagent

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blanks, is added 1 ml. of a 1 per cent solution of phthalic anhydride in 95 per cent ethyl alcohol. The solutions are then mixed by agitation, and the alcohol is removed by heating on a steam bath. Subsequently, the tubes are heated in a $148^{\circ} \pm 2^{\circ}$ oven¹ for 10 minutes, plugged with absorbent cotton which serves as a drying agent,² reheated for another 10 minutes, and placed in a desiccator which is then evacuated on a water pump.

0.5 ml. of a solution of zinc chloride in absolute methanol (12 gm. in 25 ml.³) is added to each of the thoroughly dried tubes, which are then re-plugged at once. A burette protected against moisture is used for the dispensing of the zinc chloride solution. The zinc chloride should be dried previously under a vacuum at 110° over phosphorus pentoxide for 6 hours and kept in a tightly closed bottle in a desiccator containing phosphorus pentoxide. The tubes are shaken on a shaking machine and then heated on a steam bath. The heating period should be as brief as possible, the time required being determined by that necessary to evaporate simultaneously 0.5 ml. of methanol in a plugged tube. Since the cotton plugs absorb the alcohol during the evaporation of methanol, they should be replaced by dry ones at this point in the procedure. These are then retained until the end of the experiment.

The tubes are now heated for 3 hours in a 148° oven kept dry with phosphorus pentoxide. After the tubes are cooled, 1 ml. of absolute methanol is added to each one, following which the tubes are shaken for 5 minutes on a shaking machine. At this time the reaction mixture is dissolved in one of two solvent combinations, either of which brings out the fluorescence of the phthaleins, *viz.* methanol-HCl and methanol-acetic acid.

If the methanol-HCl solvent combination is used, the foregoing dilution is accomplished by previously setting up tubes containing 9 ml. volumes of absolute methanol. Each of the sample tubes is then diluted and read *one at a time* in order to make consecutive determinations under the same experimental conditions. The first step in the foregoing procedure is to place a footed stirring rod in the reaction tube containing the 1 ml. solution of the phthalein that has been diluted with 9 ml. of absolute methanol. 1 drop of concentrated HCl is then added from a dropping bottle, and the liquids are stirred for about 15 seconds. The mixture is poured into a cuvette, and the fluorescence is read *without delay*.

If the methanol-acetic acid solvent combination is used, the dilution is

¹ The air of the oven should be kept dry with phosphorus pentoxide.

² The cotton should be oven-dried for 2 hours at a temperature between 105 – 115° .

³ The bottle and graduated cylinder to be used for the preparation of the zinc chloride solution should be dried initially in the oven and allowed to cool in a desiccator. During the first few minutes after methanol is added to zinc chloride, the bottle cannot be stoppered because of the excessive heat generated. Consequently it is covered temporarily with a small beaker containing a layer of dried absorbent cotton.

made with 9 ml. of glacial acetic acid instead of absolute methanol. In this procedure no HCl is used. Although the methanol-acetic acid solvent

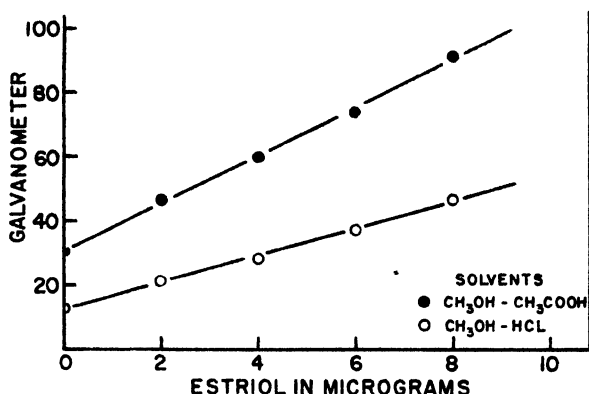


FIG. 1. Experiment 1, Table II. Relationship between the galvanometer readings and the concentration of the estradiol assayed expressed in micrograms per 10 ml. of solvent. Throughout the charts these readings are the average of quadruplicate values obtained when the instrument was set to give a reading of 65 for 10 ml. of an aqueous solution of sodium fluoresceinate containing 0.035 γ per ml.

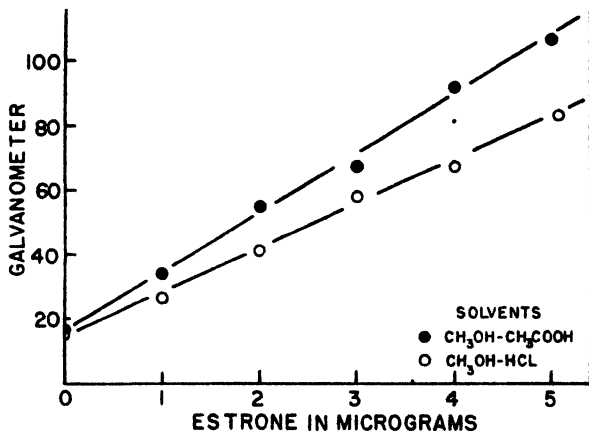


FIG. 2. Experiment 2, Table III. Relationship between the galvanometer readings and the concentration of the estrone assayed expressed in micrograms per 10 ml. of solvent.

combination gives higher values than does the methanol-HCl, it is less desirable because of the irritating fumes.

The fluorescence is measured by the Coleman Universal photofluorometer equipped with lamp Filter UV-2 and photocell Filter PC-2.

Results—Figs. 1 to 3 illustrate the linear relationship of fluorescence to

concentration for the three estrogens in both solvent combinations. The data in Table I show the accuracy and reproducibility of the method as applied to the three estrogens studied. Tables II to IV establish the validity and reproducibility of the results obtained for a given concentration *in a single experiment* in which the methanol-HCl solvent combination is used. They also demonstrate the necessity of determining a standard curve for each set of samples analyzed.

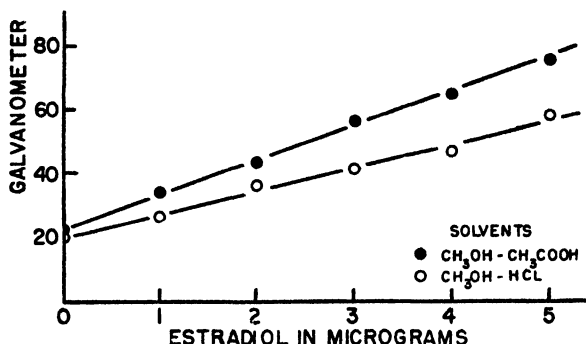


FIG. 3. Experiment 1, Table IV. Relationship between the galvanometer readings and the concentration of the estradiol assayed expressed in micrograms per 10 ml. of solvent.

TABLE I
Reproducibility of Data As Indicated by Mean Galvanometer Deflections per Microgram of Estrogen

Estrogen	MeOH-AcOH solvent			MeOH-HCl solvent		
	No. of determinations	Mean galvanometer deflection* per microgram	Standard error of mean	No. of determinations	Mean galvanometer deflection* per microgram	Standard error of mean
Estrinol	32	7.82	±0.22	32	4.26	±0.24
Estrone	39	19.48	±0.46	40	11.99	±0.56
Estradiol	80	13.90	±0.27	59	9.98	±0.08

* Value for the sample minus the average blank value.

Although the acid used in the solvent is essential for intensifying the faint primary fluorescence of the estrogen phthaleins, it also has a destructive action on the fluorescence, which subsequently negates the initial effect. Since the fluorescence of these substances disappears fairly rapidly even in the dark, it has been established that it is stable long enough to yield reproducible results if the above technique is carried out precisely. Moisture does not affect the final solution, but it must be obviated during the synthesis.

Significance of Results—Several other assay methods are in use which depend upon the fluorescence developed between the estrogens and phos-

TABLE II
Fluorescence of Product of Reaction of Estriol with Phthalic Anhydride and Zinc Chloride

Experiment No.	Concentration	Photofluorometer readings				
		Quadruplicate samples				Average
	γ					
	0	13	12	13	12	12.5
	2	20	22	20	21	21
	4	28	28	28	27	28
	6	36	37	37	38	37
	8	46	46	48	46	46.5
	0	15	13	13	14	14
	2	22	22	22	20.5	22
	4	28.5	28.5	31	31	30
	6	41	41	44.5	39	41
	8	58	63	57	56	58.5

Fluorescent standard set at a reading of 65. MeOH-HCl used as solvent.

TABLE III
Fluorescence of Product of Reaction of Estrone with Phthalic Anhydride and Zinc Chloride

Experiment No.	Concentration	Photofluorometer readings				
		Quadruplicate samples				Average
	γ					
1	0	18	18	18	18	18
	1	24	28	26	27	26
	2	42	39	37	39	39
	3	53	54	54	53	53.5
	4	69	68	68	67	68
2	5	76	73	73	76	74.5
	0	15.5	15.5	13	15	15
	1	27	27	25	27	26.5
	2	40	40	41.5	41.5	41
	3	58	58	56	59	58
	4	59	71	67	71	67
	5	86	88.5	86	71	83

Fluorescent standard set at a reading of 65. MeOH-HCl used as solvent.

phoric (3) or sulfuric acid (4, 5). These methods are known to give fluorescent products with substances other than estrogens. The present work based on a condensation of phthalic anhydride with phenols was under-

taken with the view of developing a method which would give no background fluorescence other than that due to the reagents when applied to biological extracts. The latter is now being investigated. Observations in this laboratory suggest that the specificity of the phthalic anhydride-zinc chloride test for phenolic steroids (estrogens) might be increased further by developing it as a colorimetric test.

TABLE IV
Fluorescence of Product of Reaction of Estradiol with Phthalic Anhydride and Zinc Chloride

Experiment No.	Concentration	Photofluorometer readings				
		Quadruplicate samples				Average
1	γ					
	0	19	21	19	19	19.5
	1	27	25	28	25	26
	2	37	35	36	37	36
	3	40	41	40	42	41
	4	46	47	45	48	46.5
2	5	60	60	61	50	58
	0	13	13	12	13	13
	1	19.5	*	21	21	20
	2	36	36	36	30	34.5
	3	40	43	45.5	40	42
	4	56	53	52	53	53.5
3	5	56	64	62	60	60.5
	0	9	10	9	9	9
	1	24	24	23	25	24
	2	36	36	34	22.5	32
	3	50	49	45	50	48.5
	4	62.5	61	62	55	60
	5	75	65	80.5	70	73

Fluorescent standard set at a reading of 65. MeOH-HCl used as solvent.

* Lost..

Application of Phthalic Anhydride-Zinc Chloride Reaction to Assay of Steroids Other Than Estrogens, with Special Reference to Cholesterol

Experimental Procedure—10 γ samples of androsterone, dehydroisoandrosterone, pregnanolone, 17-hydroxycorticosterone, cholesterol, and a 5 γ sample of phenol were assayed by the procedure described above, with the MeOH-HCl solvent combination. Standardization curves for cholesterol were determined by this method.

Results—The fluorescence of the acidified reaction products of phenol and 17-hydroxycorticosterone with phthalic anhydride and zinc chloride

was found to be equal to that of the reagent blanks (Table V). The fluorescence of the acidified reaction product of pregnanolone similarly produced was slightly above that of the blanks, whereas androsterone, dehydroisoandrosterone, and cholesterol exhibited appreciable readings above the background values. Fig. 4 and Table VI illustrate the linear

TABLE V

Fluorescence of Product of Reaction of Certain Neutral Steroids and Phenol with Phthalic Anhydride and Zinc Chloride

Compound tested	Photofluorometer readings				
	Quadruplicate samples				Average
Reagent blank.....	12	12	12	12	12
Androsterone.....	31	33	33	33	32.5
Dehydroisoandrosterone.....	46	38	42	38	41
Pregnanolone.....	19	19	20	19	19
Cholesterol.....	44	42	41	35	40.5
17-Hydroxycorticosterone.....	13	13	13	13	13
Phenol.....	12	12	14	11	12

Fluorescent standard set at a reading of 65. MeOH-HCl used as solvent.

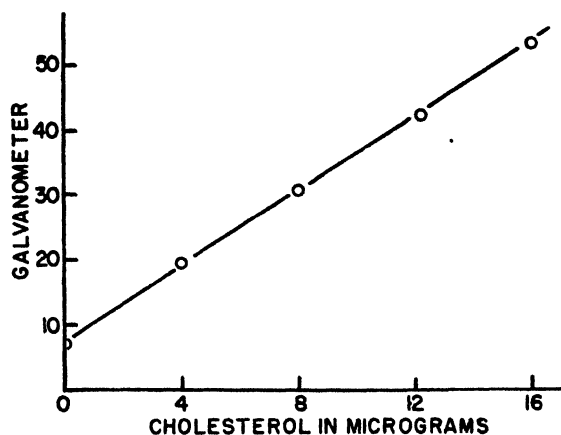


FIG. 4. Relationship between the galvanometer readings and the concentration of the cholesterol assayed expressed in micrograms per 10 ml. of solvent.

relationship of fluorescence to concentration of cholesterol by use of the MeOH-HCl solvent combination.

Significance of Results—Although Kleiner (1) states that cholesterol, androsterone, and dehydroisoandrosterone do not produce a *colored product* with phthalic anhydride, the foregoing results have revealed that androsterone, dehydroisoandrosterone, and cholesterol yield *fluorescent material*

when treated with phthalic anhydride and zinc chloride. It may be that the fluorescence given by the androgenic steroids in question can be used for a more sensitive assay than is now available for these compounds. This is under investigation. In any event, since it is possible to separate quantitatively the urinary estrogens from the neutral steroids (6), it is feasible

TABLE VI
Fluorescence of Product of Reaction of Cholesterol with Phthalic Anhydride and Zinc Chloride

Experiment No.	Concentration	Photofluorometer readings				
		Quadruplicate samples				Average
1	7					
	0	14	14	14	14	14
	2	21	21	21	20	21
	4	25	22	27	27	25
	8	36	33	33	36	34.5
2	15	48	48	46	47	47
	0	17	17	16	17	17
	1	21	23	23	23	22.5
	3	27	28	30	30	29
	10	57	50	49	51	52
3	16	66	68.5	65	61.5	65
	0	11	11	10	12	11
	4	26	23	25	27	25
	8	30	30	39	38	39
	12	51	52	50	46	50
4	16	56	54	58	58	56.5
	0	6	7	7	7	7
	4	19	20	20	19	19.5
	8	29	31	31	31	30.5
	12	43	42	41	41	42
	16	53	*	52	53	53

Fluorescent standard set at a reading of 65. MeOH-HCl used as solvent.

* Lost.

to use this reaction for the assay of urinary estrogens. The application of this test to the determination of cholesterol is now being studied.

The phenomenon of fluorescence produced by the phenolic steroids such as estrogens with phthalic anhydride is believed to be due to the formation of phthaleins (2). However, this type of condensation cannot account for the fluorescent products formed by the interaction of the non-phenolic steroids studied with phthalic anhydride. On the basis of the five non-phenolic steroids investigated, there was no apparent correlation between molecular structure and the production, or lack of production, of fluores-

cent material. Thus, there is no indication at present of a specific relation between the functional groups of the non-phenolic steroids studied and their ability to give fluorescent products when heated with phthalic anhydride and zinc chloride.

SUMMARY

1. A fluorometric method for the quantitative determination of natural estrogens has been described. It is based on the interaction of phenolic steroids with phthalic anhydride.

2. The fluorescence obtained from certain neutral androgenic and non-androgenic steroids indicates that the phthalic anhydride-zinc chloride reaction may be applicable to the assay of steroids other than estrogens. This has been proved for cholesterol.

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A MICROSPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF SUCCINIC DEHYDROGENASE*

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The method used by Potter and others (1, 2) for studying the effect of various inhibitors and activators on cytochrome-reducing enzymes has been successfully adapted to the assay of the succinic dehydrogenase content of rat tissues. The rate of reduction of oxidized cytochrome *c* by the enzyme is measured in the presence of sodium succinate. Sodium cyanide is added to prevent the reoxidation of the reduced cytochrome *c* by cytochrome oxidase, which is invariably present in the tissue being analyzed.

By the use of a micromodification, it has been found possible to measure the succinic dehydrogenase content of as little as 20 γ (wet weight) of an active tissue such as heart.

Materials and Methods

0.5 M sodium succinate (Merck) dissolved in 0.17 M phosphate buffer, pH 7.4.

m/33 sodium cyanide dissolved in 0.17 M phosphate buffer, pH 7.4.

Cytochrome *c*-salt solution, made by mixing the following solutions: (a) 0.5 ml. of 1×10^{-4} M cytochrome *c*¹ in 0.17 M phosphate buffer, pH 7.4; (b) 2 ml. of water; (c) 0.3 ml. of a water solution containing 4×10^{-3} M aluminum chloride and 4×10^{-3} M calcium chloride.²

Macromethod—The tissue was homogenized for 2 minutes by hand in a glass tissue homogenizer with 5 volumes of ice-cold m/30 phosphate buffer, pH 7.4. The homogenate was further diluted with the same buffer as

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¹ Purchased from Wyeth, Inc., and stored at -30° . Molecular weight assumed to be 14,000. The purity was over 90 per cent when assayed spectrophotometrically, from the extinction data of Horecker and Heppel (3).

² The cytochrome-salt solution should be mixed immediately before use, because the addition of the aluminum and calcium salts to the phosphate buffer results in the formation of a colloidal precipitate. Although some of the cytochrome *c* is adsorbed, this precipitation has no effect on the assay. If it can be shown that Al^{+++} and Ca^{++} do not influence the activity of the tissue being assayed (see the discussion), these salts may be omitted and precipitation thus avoided.

indicated and 0.02 or 0.04 ml. was pipetted into 0.1 ml. of the sodium succinate solution contained in a bottom corner of a Beckman spectrophotometer cuvette of 1 cm. depth.³ 2 minutes later 0.1 ml. of the sodium cyanide was added and the reaction was initiated by the addition of 2.8 ml. of the cytochrome *c*-salt solution. The cuvette was then transferred to the spectrophotometer and the reduction of cytochrome *c* was followed by observing the increase in extinction at 550 $m\mu$ with a slit width of 0.02 mm. Readings were taken every 30 seconds for a period of 3 minutes. At the end of this time a few grains of sodium hydrosulfite were added and the extinction determined. This reading represents complete reduction of the cytochrome *c*.

The final concentrations of the components of the reaction mixture are as follows: sodium succinate 0.017 M, sodium cyanide 0.001 M, aluminum chloride 4×10^{-4} M, calcium chloride 4×10^{-4} M, cytochrome *c* 1.7×10^{-5} M, phosphate buffer 0.04 M.

The reduction of cytochrome *c* proved to be a first order reaction. The optical density at any given time was subtracted from that of the completely reduced sample and the logarithm of this difference was plotted against time. The slope of this line is a measure of the drop in the concentration (moles per liter) of oxidized cytochrome *c* per unit time⁴ and is proportional to the enzyme activity. In Table I, the tabulated "enzyme

³ Since this paper was submitted for publication, it has been found that higher enzyme activities can be obtained if the tissue is homogenized in a buffered solution of sodium succinate. The method is thereby simplified; since the tissue can be greatly diluted without denaturation, larger volumes can be pipetted.

⁴ The concentration of oxidized cytochrome *c* is calculated from the equation

$$[\text{Ferricytochrome } c] = (D_r - D_t)/1.96 \times 10^4 \text{ moles per liter}$$

where D_r is the density reading upon complete reduction, D_t is the reading at any time T during the course of the reaction, and 1.96×10^4 is the difference between the molecular extinction coefficients for reduced and oxidized cytochrome *c* at 550 $m\mu$ (3). The logarithm of the concentration of ferricytochrome *c* (moles per liter) is given by the equation

$$\log [\text{ferricytochrome } c] = \log (D_r - D_t) - \log 1.96 \times 10^4$$

The slope of the line formed by plotting the values thus obtained is given by the equation

$$\frac{\Delta \log [\text{ferricytochrome } c]}{\Delta T} = \frac{[\log (D_r - D_{t_2}) - \log 1.96 \times 10^4] - [\log (D_r - D_{t_1}) - \log 1.96 \times 10^4]}{T_2 - T_1}$$

which simplifies to

$$\frac{\Delta \log [\text{ferricytochrome } c]}{\Delta T} = \frac{\log (D_r - D_{t_2}) - \log (D_r - D_{t_1})}{T_2 - T_1}$$

Thus calculation of the molar concentrations of ferricytochrome *c* for each reading is unnecessary.

activity" is equal to the decrease in the logarithm of the concentration of oxidized cytochrome *c* per minute ($\Delta \log$ [ferricytochrome] per minute) when the final tissue concentration is 1:4500 (0.02 ml. of a 1:30 brain homogenate diluted to 3 ml. final volume). Each figure represents the average of the slope obtained with a 1:4500 tissue concentration and one-half that obtained with a 1:2250 tissue concentration. The data in Table III were obtained by dividing this "enzyme activity" by the mg. of protein added. All experiments were run at room temperature (approximately 25°) unless specifically noted.

Micromodification—The reagents used in the micromodification are the same as in the macro except that the volumes are reduced by a factor of 10-fold or more. 10 μ l. (0.01 ml.)⁶ of the sodium succinate solution are pipetted into the bottom of a micro test-tube 8 \times 50 mm. 2 or 4 μ l.⁶ of the enzyme are added to the bottom of the tube and gently mixed with the succinate.⁸ This small volume is measured with a 2 μ l. capillary braking pipette (4, 5). This pipette which has a reproducibility of 1 to 2 per cent is constructed by cementing a capillary tube of 0.16 to 0.18 mm. in inside diameter and 20 to 25 mm. in length into a larger tube. After 2 minutes, 10 μ l. of the sodium cyanide solution and 280 μ l. (0.280 ml.)⁷ of the cytochrome-salt solution are added. The reactants are mixed by gentle rotation and immediately transferred, by means of a finely drawn eye dropper, to the micro cells of the Beckman spectrophotometer (10). These cells are 2.5 mm. wide, have an optical path of 10 mm., and require between 50 and 100 μ l. of solution. Zero time was taken at the moment of addition of the cytochrome *c* solution, and the extinctions were followed at 550 m μ with use of a slit width of 0.08 mm. and the pin hole attachment for the Beckman spectrophotometer (10). Calculations were carried out as in the macromethod.

It will be noted that the final volume of the reaction mixture (300 μ l.) is considerably greater than the minimum required for use in the Beckman micro cells. Although the method herein described has sufficient sensitivity for our immediate needs, it could be increased several fold by further reducing the amounts of solutions used.

EXPERIMENTAL

Effect of Varying Concentrations of Components of System

Sodium Succinate—When the final concentration of sodium succinate was varied between 0.009 M and 0.034 M, there was no change in the rate of

⁵ The capillary braking pipette (4, 5), the Levy constriction pipette (6, 7), the Kirk micro pipette (8), or the syringe burette (4, 9) may be used for this purpose.

⁶ 1 μ l. = 0.001 ml., or 1 c.mm., or 1 λ .

⁷ Measured with syringe burette (4, 9).

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reduction of cytochrome *c*. Therefore 0.017 M sodium succinate, as recommended by Potter and Schneider (2), was used. In the absence of succinate, cytochrome *c* was reduced to a slight degree. However, this reduction is fully accounted for by the action of the sodium cyanide present (see below).

Cytochrome c—A final concentration of 1.7×10^{-5} M cytochrome *c* was used, since this concentration gives suitable density readings. As might be expected from the fact that the reaction is first order, variations of the final concentration between 1.2×10^{-5} M and 1.9×10^{-5} M had no effect on the value of $\Delta \log$ ferricytochrome per minute.

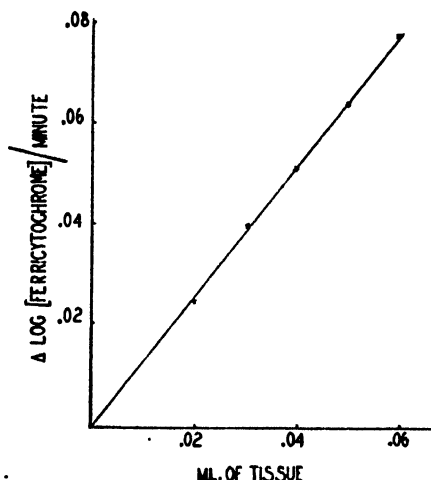


FIG. 1. Effect of varying the concentration of tissue (1:30 rat brain homogenate). Experiment by the macromethod.

Phosphate Buffer—Variations of the concentration of phosphate buffer between 0.01 M and 0.2 M did not affect the rate of reduction of cytochrome *c*.

Aluminum Chloride and Calcium Chloride—Although aluminum and calcium chlorides have been reported to increase the activity of succinic dehydrogenase (2, 11), the addition of these salts in concentrations of 4×10^{-4} M did not affect the activity of our test system (rat brain homogenate). In nineteen determinations on five rats in the absence of Al^{+++} and Ca^{++} , the average value for the enzyme activity was found to be 0.024. An equal number of determinations on the same five rats in the presence of these salts gave a value of 0.022. Nevertheless, for reasons to be discussed below, it was decided to include the salts in the assay system.

Tissue—Fig. 1 demonstrates that the rate of reduction of cytochrome *c* is proportional to the tissue concentration.

Cyanide—Variation in the sodium cyanide concentration between 0.0005 *M* and 0.002 *M* did not change the rate of reduction of cytochrome *c*. Hence a concentration of 0.001 *M*, as recommended by Potter and Schneider (2), was used. If the cyanide was omitted, no reduction of cytochrome *c* was observed. This effect is probably due to the rapid reoxidation of reduced cytochrome *c* by the cytochrome oxidase present in the tissue homogenates.

Reaction of Sodium Cyanide and Cytochrome c

In the absence of enzyme and succinate, it was found that cyanide reduces cytochrome *c* ($\Delta \log$ ferricytochrome per minute = 0.003 to 0.005). Tests with numerous different samples of cyanide, including a recrystallized sample, suggest that it is the cyanide itself which reduces the cytochrome *c*. This reduction apparently stops after 15 to 20 minutes and the addition of more cyanide at this point does not cause further reduction, although there is an ample amount of oxidized cytochrome *c* still remaining.

The cessation of this reaction may be due in part to the formation of a complex between oxidized cytochrome *c* and cyanide. Potter (12) and Horecker and Kornberg (13) have reported such complex formation and our work confirms the observation that there is a loss of enzymatically reducible cytochrome *c* when the latter is incubated with cyanide. At 37° this loss has been found to be on the order of 80 per cent after a 30 minute incubation period prior to addition of the tissue. Since the rate of complex formation is slow at 25° (half time of formation being 1 hour (13)), and since the time required for the assay is only 3 minutes, it is felt that the formation of this complex has a negligible effect on the rate of reduction under the conditions used.

Consideration of Fig. 1 shows that the straight line obtained by plotting enzyme activities against increasing amounts of tissue is extrapolated back to the origin, thus indicating that at zero enzyme concentration there is no significant reduction of the cytochrome *c*. These data would therefore suggest that, under the conditions of the assay, sodium cyanide does not cause significant reduction of cytochrome *c*. It is therefore unnecessary to make any corrections for reduction of cytochrome *c* by cyanide.

Effect of Temperature on Reaction

Table I shows that the reaction rate is increased with increasing temperature (25–37°). In these experiments the cuvettes (as well as all re-

actants, except the enzyme) were kept in a constant temperature bath until they were transferred to the spectrophotometer which was at room temperature.

In spite of the more rapid reduction rate at 37°, it was decided to run the assay at room temperature, since there is a lower rate of complex formation between cyanide and cytochrome *c*. In addition, when the micromethod was used, greater difficulties were encountered in maintaining the temperature at 37° during the procedure.

The temperature of the room was maintained at 25° ± 1°. Although errors of about 10 per cent per degree are caused by variations of room temperature, these may be minimized by running comparative experi-

TABLE I

Effect of Temperature on Enzyme Activity

Experiments carried out with 1:30 rat brain homogenate.

Temperature	Enzyme activity*
°C.	
25	0.014
30	0.020
37	0.038

* Δ log [ferricytochrome *c*] per minute with a final tissue concentration of 1:4500.

ments at the same time. When this is not possible, the results may be compared with a standard succinic dehydrogenase preparation run as a control each day.

Effect of Order of Addition of Components

Potter and Albaum (1) recommend that a mixture of sodium cyanide and sodium succinate be the last solution added to the cuvette. By following this procedure, the cytochrome oxidase contained in the tissue preparation has an opportunity to oxidize any of the added cytochrome *c* which may be present in the reduced state. We found, however, that there is a progressive loss of enzyme activity when rat brain homogenate is diluted by the cytochrome-salt mixture. After standing 5 minutes the activity was only 44 per cent of the original. This inactivating effect is further enhanced by shaking prior to the addition of succinate (70 per cent decrease). This loss can be prevented by adding the enzyme to the sodium succinate 2 minutes before the addition of the other components. The presence of the sodium succinate apparently prevents the inactivation of the enzyme, which has been observed to take place at high dilution.³

Comparison of Micro- and Macromethods

It was found that the enzyme activities as measured by the micro- and macromethods are in good agreement, as shown in Table II.

TABLE II
Comparison of Micro- and Macromethods

Tissue	Initial tissue dilution	Enzyme activity corrected for dilution*							
		Rat 1		Rat 2		Rat 3		Rat 4	
		Micro	Macro	Micro	Macro	Micro	Macro	Micro	Macro
Lung.....	1:10	0.41	0.48	0.45	0.40	0.77	0.69	0.58	0.58
Heart.....	1:100	4.7	4.1	3.1	3.8	4.0	3.6	4.0	3.8
Liver.....	1:75	3.08	3.45	2.3	2.4	2.47	2.47	3.0	3.52

* Calculated by multiplying the observed enzyme activity by the dilution factor given in the second column. These figures thus represent the enzyme activity when the final tissue concentration is 1:150.

TABLE III
Succinic Dehydrogenase Content of Rat Tissues

Tissue	Dilution added to cuvette	No. of rats	Average enzyme activity per mg. protein*	σ †
Kidney.....	1:80	10	138	38.3
Heart.....	1:100	9	97.9	19.8
Liver.....	1:75	10	66.4	9.6
Brain.....	1:20	10	19.2	5.89
Lung.....	1:10	10	16.2	3.49
Striated muscle‡.....	1:10	10	16.1	4.37

* Protein determined by micro-Kjeldahl method.

† $\sqrt{\Sigma(\text{deviation from mean})^2/(\text{number of rats})}$.

‡ Rectus abdominis.

Application of Method

Six tissues from each of ten rats* were examined for their succinic dehydrogenase content. The results are shown in Table III. Kidney was found to have a significantly higher content of succinic dehydrogenase than heart ($P = 0.004$), which in turn has a significantly greater activity than liver ($P < 0.0001$). Although liver has significantly more succinic dehy-

* The rats were killed by decapitation.

drogenase than brain ($P < 0.0001$), the differences between brain, lung, and striated muscle are not statistically significant ($P > 0.10$).

DISCUSSION

Three methods for the assay of succinic dehydrogenase are in common use. In one method the oxygen uptake of tissue is measured with succinate as a substrate (14). Since the oxygen uptake depends not only upon the concentration of succinic dehydrogenase, but on the cytochrome *c* and cytochrome oxidase contents as well, one cannot be certain that the succinic dehydrogenase is limiting in all tissues under all conditions, for although cytochrome *c* can be added in excess, cytochrome oxidase cannot. Schneider and Potter (14) attempted to circumvent this difficulty by concomitantly determining the cytochrome oxidase QO_2 with ascorbic acid as a substrate. Although they found that cytochrome oxidase was in excess in all tissues examined, the assumption that the turnover rate of the oxidase is identical under the two different assay conditions is not necessarily correct. Further, in certain pathological conditions, such as chromatolytic nerve, in which succinic dehydrogenase data are desirable, cytochrome oxidase is known to be deficient (15).

A second manometric method involves the substitution of a dye such as methylene blue for the cytochrome portion of the chain of respiratory enzymes. However, Potter has shown (16), and we have confirmed, that the rate of oxygen uptake when methylene blue is used is also dependent upon the amounts of the cytochromes present in the tissue. If the cytochrome system, when working at its maximal activity, has a faster turnover rate than does methylene blue, then the addition of methylene blue would result in a decreased activity because of the competition between the methylene blue and the cytochromes. On the other hand, if there were a deficiency in the cytochrome system great enough to make it limiting, then the addition of methylene blue would enhance the activity of the system.

In the third method the anaerobic rate of reduction of a dye such as methylene blue (17, 18), or triphenyltetrazolium chloride (19), is measured. This method is perhaps the most acceptable from a theoretical standpoint, since the cytochrome system is not active under anaerobic conditions. This reaction may also be carried out aerobically, if sufficient cyanide is added to inhibit the cytochrome oxidase. If the cytochrome system is not inactivated, a variable portion of the electron transport will proceed through this system rather than through the dye and the rate of dye reduction will then be dependent upon the concentration of the cytochrome system as well as that of succinic dehydrogenase. While the method herein described is similar to the last, it offers the advantages that the natural

biological reactant of succinic dehydrogenase, *i.e.* oxidized cytochrome *c*, is used and that it has a greater sensitivity. While it is felt that the addition of cyanide is inherently undesirable, microtechniques are not sufficiently developed to permit the reaction to be carried out anaerobically.

The observed reduction of cytochrome *c* by sodium cyanide need not present a problem. It has been ignored in the calculations, since it was shown that it either does not occur concomitantly with enzymatic reduction or it occurs so slowly that it is negligible. Whether this is simply a competitive effect due to the difference in the rates of the enzymatic and non-enzymatic processes or due to a combination of the cyanide with the cytochrome oxidase and other proteins is unknown.

The effect of the addition of aluminum and calcium chlorides was tested because of the reports that these salts increase the activity of the succinic dehydrogenase system by preventing the accumulation of inhibitory substances (2, 11). At least one of these inhibitors, oxalacetic acid, can arise as a result of the presence of diphosphopyridine nucleotide (20). It has been postulated that the calcium ion, by means of its activation of diphosphopyridine nucleotidase, hastens the destruction of this coenzyme and thereby prevents the accumulation of oxalacetate (11).^{*}

If this explanation is correct, it would seem that our inability to detect an effect of Ca^{++} or Al^{+++} on brain either at 25° or at 37° may be due to prior destruction of diphosphopyridine nucleotide during the homogenization. This conclusion seems reasonable in view of the fact that rat brain is known to have a very active nucleotidase (21). However, it was decided as a precautionary measure to use these salts with all new tissues examined until a lack of effect was definitely established.

The failure to demonstrate any changes in the activity of the system by varying the phosphate concentration confirms the report of Keilin and Hartree (18) who found the phosphate concentration had no effect on the rate of reduction of methylene blue by succinic dehydrogenase, although it did affect the succinoxidase system as a whole.

It must be borne in mind that although we speak of this as an assay for "succinic dehydrogenase" we are actually measuring the enzyme or enzyme complex which catalyzes the reduction of cytochrome *c* by succinic acid. That there may be more than one enzyme involved is indicated by work from many sources (22-28).

It is therefore not surprising that, although an analysis of the "succinic dehydrogenase" content of different rat tissues as determined by three different methods gives the same relative order of activity, the exact ratios of these activities are not constant. The methods tested were the

^{*} This explanation has recently been challenged by Keilin and Hartree (18).

aerobic oxidation of succinate in the presence of cytochrome *c*, the reduction of cytochrome *c* as described herein, and the aerobic oxidation of succinate in the presence of methylene blue and cyanide. In the aerobic oxidation of succinate in the presence of cytochrome *c*, the enzymes which both reduce and oxidize cytochrome *c* are required, whereas in the method described herein only those enzymes involved in the reduction are necessary. Furthermore there is evidence (28) that one less factor is required for the reduction of methylene blue than is the case for cytochrome *c*. Thus, depending upon which factor is limiting in a particular tissue, the relative activity may vary widely when tested with the different methods. It may further be expected that an analysis of different tissues by these three methods may yield valuable information as to the distribution of these components. This problem is under investigation.

SUMMARY

Macro- and microspectrophotometric methods have been developed for the assay of succinic dehydrogenase of animal tissues based upon the rate of reduction of cytochrome *c* in the presence of succinate.

The micromethod can be carried out with as little as 20 γ of heart tissue or 100 γ of brain tissue (wet weight) in a final volume of 300 μ l.

The effects of various factors on the rate of the reaction have been studied and various rat tissues have been assayed for their succinic dehydrogenase content.

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SULFHYDRYL PROTECTION AGAINST DEHYDROASCORBIC ACID DIABETES*

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Dehydroascorbic acid has been shown to produce diabetes (1, 2). In this respect it is similar to alloxan (3). Whereas alloxan has not yet been shown to occur physiologically, dehydroascorbic acid is accepted as occurring under physiological conditions in the cells of animals (4). Therefore, it is important to study the factors that are known to affect alloxan diabetes in order to determine their effect in dehydroascorbic acid diabetes.

Naturally occurring sulfhydryl compounds, such as glutathione, are believed to exert a protective effect physiologically against diabetogenic compounds (5). Compounds such as cysteine (6), glutathione (6), and 2,3-dimercaptopropanol (BAL) (7) given before an injection of alloxan will protect against the diabetogenic effects of alloxan. When the same substances are injected after alloxan, there is no protection against diabetes. Therefore, the effects of sulfhydryl compounds on the diabetogenic action of dehydroascorbic acid were studied.

EXPERIMENTAL

Dehydroascorbic acid was prepared as previously described (2). The sulfhydryl compounds were administered as aqueous solutions of the following concentrations: cysteine (free base) 10 per cent; neutralized glutathione (GSH) 10 per cent; and 2,3-dimercaptopropanol (BAL) 0.67 per cent.

All substances were administered intravenously to male Sprague-Dawley rats weighing between 100 and 200 gm. A preliminary desensitizing dose (2) of dehydroascorbic acid (0.2 gm. per kilo) was given to all rats on the 1st day of injection only.

A control group (A) was given dehydroascorbic acid (0.7 gm. per kilo) on 3 consecutive days. Diabetes, as defined by a hyperglycemia of at least 200 mg. per cent 1 week after the last injection, was found in 87 per cent of the rats.

A second group (B) was given sulfhydryl compounds 2 minutes before

* This investigation was supported in part by a research grant from the National Institutes of Health, United States Public Health Service.

each main injection of dehydroascorbic acid. Although the daily dose of various sulfhydryl compounds varied from 1.0 to 8.2 mm per kilo, there was complete protection against diabetes in thirty-two animals. Those animals receiving the smallest dose of sulfhydryl (as BAL) showed a slight temporary hyperglycemia about 2 to 3 days after the last injection.

A third group (C) was given sulfhydryl compounds 10 minutes after each of the three main injections of dehydroascorbic acid. In 75 per cent of the animals diabetes was produced.

TABLE I
Effect of Sulfhydryl Compounds on Production of Diabetes

Dehydroascorbic acid (0.7 gm. per kilo*) was injected intravenously on 3 successive days.

Sulphydryl compound	—SH mm per kilo per day	No of rats	Average blood sugar, mg. per cent					Per cent dia- betic
			Initial	2-3 days	4-5 days	1 wk.		
						Dia- betic	Non- dia- betic	

Group A; control								
None.	None	15	100	364	490	448	114	87

Group B; sulphydryl 2 min. before dehydroascorbic acid								
Cysteine.	8.2	13	111	122	117		102	0
GSH	3.2	8	123	134	127		117	0
BAL	1.0†	11	102	210	134		119	0

Group C; sulphydryl 10 min. after dehydroascorbic acid								
Cysteine.. . . .	8.2	12	112	403	340	360	126	75
GSH	3.2	5	122	404	282	349	155	60
BAL	1.0†	3	102	495	635	445		100

* On the 1st day a desensitizing dose of 0.2 gm. per kilo was given prior to all other injections.

† 0.5 mm of BAL.

The results obtained during the 1st week following injection are summarized in Table I. The rats were followed for a 2nd week with no change in results except in the case of three diabetic rats that had received the large dose of cysteine 10 minutes after dehydroascorbic acid. At the end of 2 weeks the blood sugars of these rats had dropped below 200 mg. per cent. At the end of 2 weeks, therefore, diabetes was found in only 50 per cent of the animals in the third group, whereas the other groups were unchanged. Table I shows the results on all rats that survived the in-

jection period. The mortality during the injection period was as follows: the control group (A), 42 per cent; the group (B) receiving sulfhydryl before dehydroascorbic acid, 26 per cent; and the group (C) receiving sulfhydryl after dehydroascorbic acid, 57 per cent.

DISCUSSION

When dehydroascorbic acid is injected intravenously following an injection of sulfhydryl compound, three reactions which tend to remove it from the blood stream can occur. First, the compound can decompose spontaneously to diketogulonic acid at a rate such that it is half destroyed in 2 minutes (8); second, it can be reduced by sulfhydryl to ascorbic acid (9); and third, it can combine with a sulfhydryl compound to give an addition product (10). These reactions are similar to those that occur with alloxan (11, 12). Diketogulonic acid (13) and ascorbic acid (14) are not diabetogenic. A combination of dehydroascorbic acid with sulfhydryl removes an active group of dehydroascorbic acid, and so the resultant combination is probably not diabetogenic.

These three mechanisms, therefore, may combine to remove dehydroascorbic acid from the blood and thus prevent an adequate concentration from reaching the β cells of the islets of Langerhans in the pancreas. It is assumed that these same mechanisms would also be effective within the cell. Similar observations with alloxan led to the hypothesis that the diabetogenic effect of alloxan was due to the inactivation of essential enzymes through combination with a sulfhydryl group (7, 15). This same hypothesis is applicable to the mechanism of action of dehydroascorbic acid.

Sulfhydryl compounds given 10 minutes after dehydroascorbic acid fail to protect the animals from diabetes. Therefore, dehydroascorbic acid must bring about the necessary alterations for the production of diabetes within a few minutes after injection. The short time required for this diabetogenic action suggests that dehydroascorbic acid acts either by rapidly destroying or combining with an essential component of cellular metabolism. If this block involves the sulfhydryl of an enzyme, as has been suggested in the case of alloxan (7), the process must involve the irreversible addition of dehydroascorbic acid to the sulfhydryl and not an oxidation of the sulfhydryl to a disulfide linkage. For one would expect a disulfide linkage to be reduced by the sulfhydryl compounds given after the dehydroascorbic acid and diabetes would thus be prevented. This is not the case. Therefore, if an enzymatic sulfhydryl group is blocked, it is probably the result of an irreversible combination with dehydroascorbic acid.

Dialuric acid and ascorbic acid are the respective reduction products of

alloxan and dehydroascorbic acid. Dialuric acid is relatively insoluble and readily oxidized; hence it is difficult to test it for diabetogenic properties. Ascorbic acid is readily soluble. Repeated large daily doses of ascorbic acid (5.0 to 6.0 gm. per kilo) given to rats in this laboratory did not produce hyperglycemia. This analogy lends further support to earlier work indicating the inability of dialuric acid to produce diabetes (15).

Sulfhydryl compounds have a similar effect in dehydroascorbic acid and in alloxan diabetes. These two types of diabetes are also similar in that there is a triphasic blood sugar response following injection (1), and in that they both respond to small doses of insulin (2). Preliminary work indicates that they produce similar histological lesions in the islets of Langerhans and liver. However, the necrosis in the former is not as marked after dehydroascorbic acid administration.

SUMMARY

Cysteine (8.2 mm of sulfhydryl per kilo), glutathione (3.2 mm of sulfhydryl per kilo), or 2,3-dimercaptopropanol (1.0 mm of sulfhydryl per kilo) given intravenously 2 minutes before the intravenous administration of 0.7 gm. per kilo of dehydroascorbic acid on 3 successive days completely prevented the development of diabetes in thirty-two rats. Of fifteen rats not receiving sulfhydryl compounds, thirteen developed diabetes. The above dose of cysteine, glutathione, or 2,3-dimercaptopropanol injected 10 minutes after dehydroascorbic acid did not prevent the development of diabetes in fifteen out of a total of twenty rats.

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THE METABOLISM OF GLYCINE BY FOLIC ACID-DEFICIENT CHICK LIVER HOMOGENATES*

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Recent work has made it clear that folic acid is intimately concerned in glycine metabolism. It was shown that a deficiency of glycine produced by feeding sodium benzoate (1) was partially prevented by this vitamin. Likewise, growth in rats fed toxic levels of glycine is improved by folic acid (2, 3). Holland and Meinke (4) have found that both glycine and folic acid seem to improve the ability of such organisms as *Streptococcus faecalis* to grow with very low levels of serine in the medium. Likewise there is some evidence that porphyrin metabolism is influenced by folic acid both in animals and in microorganisms (1, 5, 6). It is known that the α -methylene carbon of glycine is incorporated into porphyrin (7) and that the carboxyl carbon of glycine does not appear in the porphyrin portion of the hemoglobin molecule (8). These experiments taken together suggest that folic acid is necessary for the breakdown of glycine into a 1-carbon intermediate or incorporation of this carbon into serine and other substances such as purines, pyrimidines, and porphyrin. Evidence that folic acid is concerned in the production of pyrimidines and purines has been reviewed in a recent paper by Prusoff *et al.* (9), but direct evidence that the vitamin action is exerted through its influence on glycine metabolism is lacking. It seemed likely to us that the best approach to the solution of this problem was through the use of C^{14} -labeled glycine. The results of the first of these studies is presented here.

For the present experiments chicks were used, since it is possible to induce an uncomplicated dietary deficiency of folic acid in this species. The radioactive glycine¹ used contained C^{14} in the carboxyl group and exhibited an activity of about 10 μ c. per mg.

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¹ The glycine containing C^{14} in the carboxyl group was generously supplied by Dr. Martin D. Kamen of the Mallinckrodt Institute for Radiology, Washington University, St. Louis, Missouri.

EXPERIMENTAL

Day-old white rock chicks were obtained from a hatchery and housed in a metal brooder maintained at constant temperature. Food and water were given *ad libitum*. The deficient diet was similar to that commonly employed for folic acid assays with chicks and had the same composition as that used by Keith *et al.* (10). Control birds received the deficient diet supplemented with 200 γ of folic acid per 100 gm. of diet.

After 3½ weeks on the diet the chicks were sacrificed and 2 gm. samples of liver from each bird were homogenized in a Potter-Elvehjem apparatus with an equal volume of KHCO_3 -KCl buffer as described by Winnick *et al.* (11). 0.3 ml. aliquots of the homogenates were incubated with 0.1 ml. of solution containing 0.0257 mg. of carboxyl-labeled radioactive glycine (specific activity about 10 $\mu\text{c.}$ per mg.). 0.1 ml. of folic acid solution containing either 2 or 10 γ of folic acid and the same amount of Na_2HPO_4 was added to one set of duplicate tubes. An equal amount of Na_2HPO_4 solution of the same concentration was added to duplicate control tubes; the total volume in all cases was 0.5 ml. Incubations were carried out at 38° in conical centrifuge tubes supported in a horizontal position and under an atmosphere of 95 per cent O_2 with 5 per cent CO_2 . After 60 to 90 minutes the proteins were precipitated with trichloroacetic acid and extracted with alcohol, alcohol-ether, and ether, to obtain the phospholipides. The technique described by Winnick and coworkers (11) was used throughout. The trichloroacetic acid supernatants from the deficient and control chicks were saved separately and each group pooled for the isolation experiments described below. The samples containing added folic acid were not saved separately since the effect of the vitamin *in vitro* seemed to be very small.

An aliquot of the phospholipide solution from each experiment was evaporated on a counter plate and counted directly. A colorimetric phosphorus determination (12) was made on a similar aliquot after wet ashing with sulfuric acid. The protein residue was dried *in vacuo*, weighed, powdered, and counted. Corrections for self-absorption due to protein were calculated from a curve constructed by plotting counts against weight for two of the most radioactive protein samples obtained in the experiment. The same correction curve, which agrees closely with a published curve (13), was used for correcting all other sample counts, except those of the phospholipides in which the correction for sample weight was negligible.

Thymine, uric acid, adenine, xanthine, guanine, uracil, serine, glycine, guanidoacetic acid, and creatine, as carriers, were each added to separate aliquots of the trichloroacetic acid supernatants. Appropriate procedures for the isolation of each substance were used and the recovered compound recrystallized until no further loss in activity resulted. In some cases it

was necessary to remove the trichloroacetic acid by ether extraction and concentrate the residual solution by evaporation in order to provide sufficiently active material to give satisfactory counting rates. After a preliminary count each substance, with the exception of glycine and serine, was treated with 1 ml. of a solution containing 5 mg. of ninhydrin and heated to destroy any amino acids. If any loss of activity occurred, an additional treatment with ninhydrin was made. It was found that when a similar procedure was applied to a mixture of radioactive serine and non-radioactive thymine more than 95 per cent of the counts were removed and

TABLE I

Effect of Dietary Folic Acid and Folic Acid in Vitro on Uptake of Radioactive Glycine by Chick Liver Homogenate Proteins and Phospholipides

0.0257 mg. of glycine containing C^{14} in the carboxyl group and giving about 54,000 c.p.m. was added to 0.3 ml. of 1:1 homogenate. Incubation period 60 to 90 minutes at 39°; total volume 0.5 ml; counting efficiency approximately 7.5 per cent; accuracy approximately ± 3 per cent.

Diet	Folic acid addition to homogenate	No. of samples	C.p.m. per mg.	
			Average	Range
Proteins (25-30 mg.)				
Deficient	γ	13	0.55	0.34- 0.79
"	2 or 10	11	0.60	0.41- 0.86
" + 200 γ folic acid		8	1.46	0.78- 2.28
" + 200 γ " "	2 or 10	7	1.64	0.93- 3.04
Phospholipides (3-4 mg.)				
Deficient		6	10.3	7.4 -12.6
"	2 or 10	6	13.2	11.6 -15.6
" + 200 γ folic acid		6	21.2	13.1 -37.1
" + 200 γ " "	2 or 10	5	33.1	16.3 -46.6

only negligible activity remained. It appears, therefore, that the activities recorded for these compounds are not due to amino acid contaminants.

RESULTS AND DISCUSSION

The results of the counts on phospholipide and proteins are given in Table I. It may be seen that the liver homogenates from the deficient chicks showed a much lower degree of incorporation of radioactive carbon than did those from the replete birds. These results have not yet been compared with similar studies on chicks rendered deficient in other members of the vitamin B complex. However, the results given in Table II strongly

suggest that the reduced incorporation of the radioactivity in the deficient chick livers is due in part to a reduced conversion of glycine to serine. In the light of these and other experiments (4) it seems likely that folic acid is required for the conversion of glycine to serine.

The results of the carrier experiments on the trichloroacetic acid extracts are shown in Table II. While the radiochemical purity of the compounds isolated by addition of carriers has not been established as fully as may be desirable, it seems likely that the results are essentially correct. In some cases there were large differences in activity of compounds isolated by

TABLE II

Effect of Folic Acid on Metabolism of Glycine by Chick Liver Homogenates

0.0257 mg. of glycine giving 54,000 c.p.m. was added to 0.3 ml. of 1:1 homogenate. Total volume 0.5 ml.; efficiency of counting arrangement approximately 7.5 per cent; counting accuracy approximately ± 2.2 per cent.

Carrier added	Quantity of carrier per experiment	Specific activity		Per cent activity recovered	
		Deficient	Replete	Deficient	Replete
	mg.	c.p.m.	c.p.m.		
Glycine.	150	150.0	55.5	41.7	15.4
Serine.	150	85.0	131.0	23.6	36.4
Guanidoacetic acid	175	26.8	27.0	8.7	8.7
Creatine.	150	1.19	4.13	0.33	1.15
Uric acid.	150	0.32	3.13	0.09	0.87
Xanthine.	150	0.18	0.80	0.05	0.22
Adenine HCl	37.5	0.06*	0.28*	0.00	0.04
Guanine.	37.5	0.57	1.02	0.04	0.07
Thymine.	18.7, 37.5	2.41	5.80	0.08	0.40
Uracil	75	4.04	5.10	0.56	0.71
Total.				75.15	63.96
Residual activity				75.8	64.50

* Counted as the picrate.

identical procedures from the two supernatants. The same probable gross contaminants (serine and glycine) were present in both solutions. Furthermore, when the isolations were repeated with different ratios of carrier to sample, the same total amounts of activity were recovered. Within the limits of error the activity recovered from each of the two solutions was approximately equal to this residual activity. Two compounds, cytosine and hypoxanthine, were unavailable for testing and might be expected to have some activity, but probably not enough to alter the results appreciably.

It may be seen that the thymine, uracil, xanthine, and uric acid of the pyrimidines and purines tested were appreciably radioactive. With the

exception of uracil, the activities of those obtained from the livers of folic acid-fed chicks were very much greater than similar compounds from the livers of the deficient birds. These results are in accord with the supposed function of folic acid in the biosynthesis of purines and pyrimidines and provide evidence in support of these concepts.

Adenine and guanine showed only very slight activities and it may be presumed that these compounds, if formed during the incubation, were not primary condensation products. It should be noted that the ratios of activities of uracil were not greatly different from those of serine. Since the glycine probably breaks down to give bicarbonate (and "formate") in an amount approximately equivalent on a molar basis to the serine concentration, it is likely that the ratio shown by the two uracil activities is solely a consequence of the different concentrations of radioactive bicarbonate. On the other hand the activity ratios of thymine, xanthine, and uric acid do not seem to permit such a simple explanation. It therefore appears that folic acid either directly or indirectly promotes the synthesis of this group of compounds.

Examination of the data in Table II reveals that the rate of disappearance of glycine and appearance of serine was much more rapid in the liver homogenates from folic acid-replete birds than in those from the deficient chicks. The increased rate of serine production obtained in these experiments confirms the suggestion of Holland and Meinke (4) that folic acid promotes the conversion of glycine to serine.

The guanidoacetic acid production from glycine by chick liver was found to be fairly rapid and apparently unaffected by the folic acid deficiency as shown by the data in Table II. It is of interest that a second carrier experiment with guanidoacetic acid, carried out on the same supernatants a month later, showed only a fraction of the activity obtained in the first experiment. The activity of glycine carrier isolated at the later date had increased enough to account for the disappearance of the guanidoacetic acid. Since the first isolation of guanidoacetic acid was made some weeks after the incubations, it is entirely probable that the values given for this compound should be higher.

The activity of creatine was higher in the supernatants from folic acid-fed chicks. Since the guanidoacetic acid activity was essentially the same in the two, it is probable that methylation of the guanidoacetic acid must have taken place more rapidly in the livers of the replete birds. Evidence that folic acid may be involved in creatine metabolism has recently been obtained by Dinning and Day (14).

The accelerated disappearance of glycine in the incubations with livers from folic acid-fed chicks deserves comment. If the mechanism for serine formation proposed by Siekevitz and Greenberg (15) is correct, this

observation suggests the possibility that folic acid may be directly concerned with the breakdown of glycine to give "formate." According to the results of Sakami (16) it is quite likely that the methyl groups of choline are also available for "formate" production. If folic acid promotes formation of "formate" by increasing the rate of breakdown of glycine, it could thus possibly exert a sparing action on methyl donors. That folic acid and choline are interrelated has been shown by Schaefer *et al.* (17). However, evidence has been obtained that folic acid is involved in "formate" metabolism (18, 19). It seems possible that accumulation of "formate" from glycine may reduce the rate of the further breakdown of glycine and that the action of folic acid is to remove "formate" by increasing the rate of its condensation with glycine.

SUMMARY

Liver homogenates from folic acid-deficient and replete chicks were incubated with carboxyl-labeled radioactive glycine and the incorporation of the radioactive carbon into the proteins and phospholipides measured. Proteins and phospholipides of the livers from deficient chicks were found to be only one-half to one-third as active as those from the folic acid-fed chicks.

The addition of folic acid *in vitro* was found to elevate slightly, but probably not significantly, the uptake of radioactive carbon from glycine.

Carrier isolations were conducted on the supernatants after precipitation of the proteins. The results indicate that the livers from folic acid-fed birds are capable of transforming glycine to serine much more rapidly than those from deficient birds. The activities of creatine, uric acid, xanthine, and thymine isolated from livers of replete chicks were found to have much higher activities than those isolated from the livers of deficient chicks. Adenine and guanine were found to have slight or no activity, while uracil was moderately active in both preparations.

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3-HYDROXYANTHRANILIC ACID METABOLISM

III. MOLAR CONVERSION TO QUINOLINIC ACID*

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It has been demonstrated (1, 2) that an increase in the excretion of quinolinic acid occurs after the feeding of 3-hydroxyanthranilic acid to rats, and that 3-hydroxyanthranilate is converted to quinolinic acid by rat liver slices and homogenates (3, 4). Determination of quinolinic acid was made by decarboxylation with glacial acetic acid treatment and by measuring the resultant nicotinic acid microbiologically with *Lactobacillus arabinosus* as the test organism.

It was found, in the present study, that 3-hydroxyanthranilic acid fluoresces and the known products of the enzyme system do not. As a result a method has been developed to determine the amount of 3-hydroxyanthranilate metabolized during the course of incubation with the liver enzyme system. The data obtained by this method have been compared with the observed values for quinolinic acid formed. In these tests the effect of varying the amount of substrate, or of enzyme preparation added, and the time of incubation has been investigated. Other experiments have shown that the enzyme system from rat liver is stable and that acetone-dried powders prepared from rat liver are active sources of this system.

EXPERIMENTAL AND RESULTS

The fluorescence of 3-hydroxyanthranilic acid was measured at different concentrations and the fluorescence observed was found to be proportional to concentration (Fig. 1). The fluorescence was measured with the Coleman No. 12B photofluorometer (No. B-1 and No. PC-1 filters). Quinine sulfate was used as a reference standard. The linear relationship of fluorescence and concentration was observed with water, or with HCl, NaOH, borate buffer, pH 7.4, and Krebs-Ringer-phosphate buffer, pH 7.4 (0.1 M in each case), as solvents, and it was also found that the degree of

* We are indebted to Mona M. Marquette for assistance with the initial experiments, and to Merck and Company, Inc., Rahway, New Jersey, for supplying the 3-hydroxyanthranilic acid used in these studies. The quinolinic acid was purchased from the Bios Laboratories, Inc.

fluorescence varies with the pH, the maximum being at pH 7.0 to 7.4. Quinolinic acid, *N'*-methylnicotinamide, and nicotinic acid, which may be produced during the metabolism of 3-hydroxyanthranilate by the enzyme system, did not fluoresce under the conditions used for measuring 3-hydroxyanthranilic acid. Since 3-hydroxyanthranilate could be quantitatively recovered in the presence of heat-inactivated rat liver preparations, the fluorescent property was used to measure the changes in concentration of this compound effected by the enzyme system.

Liver from young adult rats was used as the enzyme source, and experiments were performed both with liver homogenates and with acetone-dried powders of liver. The homogenates were prepared in a Potter-Elvehjem homogenizer with Krebs-Ringer-phosphate buffer; the acetone powder

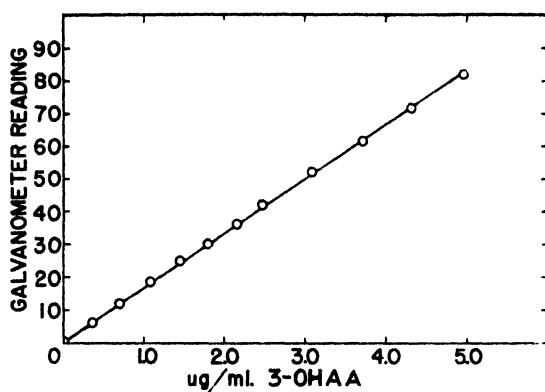


FIG. 1. Fluorescence observed with different concentrations of 3-hydroxyanthranilate in phosphate buffer at pH 7.4

preparations were made by suspending the powder in Krebs-Ringer-phosphate buffer, centrifuging, and using the clear supernatant as the enzyme solution. In all experiments, a known amount of substrate was incubated with the enzyme preparation and buffer (final pH 7.4) in test-tubes in a water bath at 37° for a given period. After incubation, the tubes were placed in a boiling water bath for 1 minute to inactivate the enzyme, the incubation mixture was filtered, and the amount of 3-hydroxyanthranilate remaining was determined. For this determination 1 ml. of filtrate was diluted with 10 ml. of buffer and the fluorescence read in the photofluorometer. Appropriate blanks for the fluorescence attributable to the enzyme preparations were made in all cases. Standard curves were prepared by adding known amounts of substrate in each series of experiments for the substrate incubated in buffer, or incubated with boiled enzyme preparations. As can be seen from Fig. 2, the conversion of the substrate to

non-fluorescent products was proportional to the enzyme concentration. With constant enzyme concentration, the conversion of substrate was proportional to the amount added until the enzyme became limiting (Fig. 3).

In the experiments with varying amounts of substrate added (Fig. 3), an acetone powder preparation (5.4 mg. added per tube) was used as the enzyme source. Similar data were obtained in other experiments when homogenized fresh liver was used. For example, when 40 mg. of homogenized liver were used per tube, the amount of substrate metabolized increased to a maximum (250 γ , or 1.63 μM in 30 minutes) when 300 to 500 γ (1.96 to 3.27 μM) of substrate were added. No conversion occurred with the use of boiled enzyme preparations. The proportionality between the

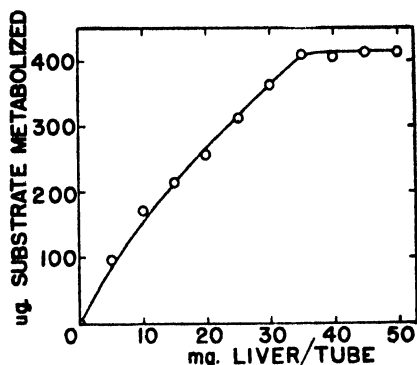


FIG. 2

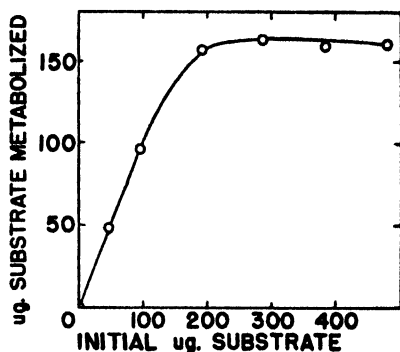


FIG. 3

FIG. 2. Effect of increased levels of homogenized liver on the amount of 3-hydroxyanthranilate metabolized as measured fluorometrically. Initial substrate concentration, 500 γ (3.27 μM) per tube; incubation time, 30 minutes.

FIG. 3. Effect of increased levels of substrate present on the amount of substrate metabolized by a constant amount of enzyme. Acetone powder was used (5.4 mg. of dry weight per tube) with an incubation time of 45 minutes.

amount of enzyme preparation used and the amount of 3-hydroxyanthranilate metabolized demonstrated that the fluorescent measurements would be useful in following the metabolism of 3-hydroxyanthranilate by this enzyme system.

Coupled with these experiments on the disappearance of 3-hydroxyanthranilate, microbiological determinations of nicotinic acid were made, with *L. arabinosus* as the test organism, before and after glacial acetic acid treatment of an aliquot of the filtrate from the heated incubation mixtures. This procedure has been found to convert effectively quinolinic acid to nicotinic acid. Quinolinic acid incubated both in the presence and absence of tissue preparations was completely converted to nicotinic acid after acetic acid treatment (100, 100.3, 100.4, 100.9, 103.2, 100.9, and 99 per

cent yield on a molar basis in individual experiments). No interference with fluorometric measurements of 3-hydroxyanthranilate was observed attributable to quinolinic acid. Corrections were made for the nicotinic acid content of the enzyme preparation. As can be seen from Table I, from 73 to 100 per cent of the 3-hydroxyanthranilic acid metabolized was

TABLE I

Effect of Substrate Concentration and Incubation Time on Metabolism of 3-Hydroxyanthranilic Acid by Rat Liver Homogenates and Acetone Powders

40 mg. of homogenized liver, fresh weight, 25.6 per cent dry weight, or 5.44 mg of acetone powder used per tube.

Substrate added	Incubation time	3-Hydroxyanthranilic acid metabolized (A)	Quinolinic acid formed (B)	(B)/(A) × 100
μM	min.	μM	μM	
0.65	5	0.65	0.58	89
0.65	5	0.65	0.52	80
0.65	30	0.65	0.60	92
0.65	30	0.65	0.54	83
1.31	5	1.12	1.02	91
1.31	30	1.21	1.13	93
1.63	5	0.94	0.83	88
1.63	30	1.21	1.15	95
1.96	5	1.32	1.11	84
1.96	30	1.73	1.51	87
2.61	5	1.32	1.08	82
2.61	30	1.65	1.47	89
3.27	5	1.25	1.05	84
3.27	30	1.62	1.49	92
3.92	5	1.24	1.04	84
3.92	30	1.63	1.46	90
6.54*	30	4.84	3.53	73
0.65†	5	0.65	0.58	89
0.65†	30	0.65	0.59	91
1.63†	5	0.72	0.72	100
1.63†	30	1.26	1.08	86

* 500 mg. (fresh weight) of liver used.

† Acetone-dried powders used as enzyme source in these experiments.

accounted for as quinolinic acid, when liver homogenates or acetone-dried powders of liver were used as the enzyme source.

Thus, with the use of the fluorometric method for following the metabolism of 3-hydroxyanthranilate, it has been possible to account for the primary product of the system, quinolinic acid, in terms of the amount of substrate metabolized. The molar ratio of the amounts of substrate me-

tabolized and quinolinic acid produced is reasonably consistent (Table I). Further, no consistent differences attributable to the initial substrate concentration or to the time of incubation were found. In previous studies (3, 4), the conversion of 3-hydroxyanthranilate to quinolinic acid was calculated on the assumption that all of the substrate was metabolized. It now appears that the conversion reported earlier may have been low, since all of the substrate was probably not metabolized.

It will be noted, however, that, as in previous experiments, the ratio of the amount of quinolinic acid produced to the total quantity of substrate present or metabolized was the lowest when high substrate concentrations (1 mg. or $6.54 \mu\text{M}$) and larger amounts of homogenized liver (500 mg.) were used. These data suggest that under the latter conditions secondary products other than quinolinic acid may be formed. This is supported by the observation (4) that the yields of quinolinic acid obtained with the lower enzyme and substrate concentrations were consistent when either 1 N H_2SO_4 or glacial acetic acid treatment was used. When nicotinic acid was determined before and after treatment of the incubation mixtures with 1 N H_2SO_4 , 8 to 20 per cent of the 3-hydroxyanthranilate metabolized (molar basis) was converted to nicotinic acid. From 10 to 22 per cent conversion of quinolinic acid to nicotinic acid was observed under identical conditions of 1 N H_2SO_4 treatment.

N'-Methylnicotinamide was also determined on aliquots of the filtrates of the incubation mixtures by the acetone-fluorometric method of Huff and Perlzweig (5), but no significant amounts of this substance were detected (less than 2 γ produced from 100 to 500 γ of substrate). Ellinger (6) and Perlzweig and associates (7) observed that nicotinamide was methylated by enzyme systems from rat liver slices, but relatively small amounts of *N'*-methylnicotinamide were produced. This was also the case when nicotinic acid was used instead of nicotinamide.

As in previous studies (4), no evidence for the formation of significant amounts of nicotinic acid (measured directly by microbiological analysis of the filtrates or after autoclaving with 1 N NaOH) by the test system has been secured. It therefore appears likely that, although *N*-methylnicotinamide and nicotinic acid may be produced by the test system, the amounts are too small to permit specific investigations on the factors influencing their synthesis.

Livers kept frozen for 2 months possessed high activity (90 to 100 per cent of the original) and homogenates refrigerated for 2 days prior to use still showed approximately 50 per cent of the original activity. Acetone-dried powders stored in a desiccator at room temperature lost about 50 per cent of their original activity in 1 month and about 95 per cent of their original activity after 3 months.

SUMMARY

The conversion of 3-hydroxyanthranilic acid to quinolinic acid by liver homogenates and acetone-dried powders of liver has been studied.

By the use of a fluorometric method, developed for the determination of 3-hydroxyanthranilate in the presence of other components of the system, it has been shown that 73 to 100 per cent of the 3-hydroxyanthranilate metabolized is converted into quinolinic acid.

Neither nicotinic acid nor *N*'-methylnicotinamide is produced by this system in significant amounts, and it is concluded that the primary product of the system is quinolinic acid.

Acetone-dried powders of rat liver are an active source of the enzyme system. Rat liver kept frozen for at least 2 months retained high enzyme activity.

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METABOLISM OF GLUTATHIONE

II. DETERMINATION OF GLUTATHIONE AND PRODUCTS OF ITS HYDROLYSIS IN BLOOD*

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A method for the determination of glutathione and the sulfur-containing products of the hydrolysis of glutathione has been developed in this laboratory. This method has been applied to tissues, enzymatic digests, and blood. This report is concerned with the description of the method for blood.

The method depends upon (1) the lability of the γ -glutamyl linkage to mild hydrolysis with acid and (2) the selective response of the method of Sullivan and Hess (1) in the determination of cysteine and cysteinylglycine (2). A combination of these factors may be utilized so that cysteine, cysteinylglycine, γ -glutamylcysteine, and glutathione may be determined by a Sullivan determination on unheated and on heated portions of a filtrate of blood.

Presumptive evidence for the presence of significant amounts of cysteinylglycine and γ -glutamylcysteine in blood has been obtained. Venous blood from the normal human has been found to have a very constant content of these materials; in certain cases of disease marked variations have been observed.

EXPERIMENTAL

Hydrolysis of Glutathione and γ -Glutamylcysteine—Concentration of acid, type of acid, and time were studied as factors in the hydrolysis of glutathione and γ -glutamylcysteine. The acid of choice was found to be phosphoric acid; the concentration of acid selected was between 0.2 and 0.5 M. The optimum time of heating was found to be 90 minutes at this altitude (a water bath boils at 94°). It is suggested that 60 minutes would be adequate at or near sea-level. Glutathione was found to be converted quantitatively to cysteinylglycine, whereas γ -glutamylcysteine was converted

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quantitatively to cysteine. Excellent recoveries of mixtures of cysteine, cysteinylglycine, and glutathione were obtained.

Application to Blood—The method for blood is as follows: Whole blood was collected with heparin as the anticoagulant (oxalate was found to be unsatisfactory) and a hematocrit was determined. 5 ml. of the whole blood were transferred to an Erlenmeyer flask and 10 ml. of water, saturated with digitonin, were added. After hemolysis was complete, 2 ml. of 50 per cent trichloroacetic acid were added dropwise with mixing; the flask was stoppered, shaken vigorously, and the mixture was centrifuged until a clear supernatant solution was obtained. 5 ml. portions of the supernatant solution were transferred to each of two colorimeter tubes (calibrated at 7 ml.), and 1 ml. of 2 M phosphoric acid was added to each tube. One tube was heated in a boiling water bath for 90 minutes and, after cooling, 1 ml. of 6 N sodium hydroxide was added to each tube. The volume was adjusted to 7 ml. and the method of Sullivan and Hess (1) was applied. The tubes were read (5 minutes after the addition of hydrosulfite) at 500, 540, and 580 $m\mu$ in the Coleman junior clinical spectrophotometer.

Calculation of Results—As has been reported (2), the reading at 540 $m\mu$ is a measure of total cysteine and cysteinylglycine. The ratio between the readings at 500 and 580 $m\mu$ is a measure of the relative concentrations of cysteine and cysteinylglycine. The 500:580 ratio for cysteine was found to be 3.4; the ratio for cysteinylglycine was 1.3. The composition of each was calculated from a graph utilizing these two values. Since all values were expressed as mg. per cent of glutathione, the standard curve was determined with solutions of glutathione. The values that were obtained were total glutathione (the 540 value of the heated tube), per cent cysteine in the heated tube (from the 500:580 ratio), cysteinylglycine plus cysteine (as glutathione) in the unheated tube, and the per cent cysteine in the unheated tube. From these values the concentration of cysteinylglycine, cysteine, glutathione, and γ -glutamylcysteine was calculated. Since no free cysteine was detected in the amount of blood used in these determinations (i.e. the ratio on the unheated tube was 1.3), only the values for cysteinylglycine, γ -glutamylcysteine, and glutathione are reported. In practice, all values are corrected to a hematocrit of 50 per cent.

The type of results obtained with the method is illustrated in Table I. Blood from five persons was analyzed over a 5 hour period to determine how much change might result from standing at room temperature. As long as heparin was used as the anticoagulant, no detectable changes were observed; therefore, each value is an average of four determinations. For purposes of comparison, reduced glutathione was estimated by an adapta-

tion of the nitroprusside method (3). Several hundred samples have been analyzed by our method and the values reported here are representative of the normal non-fasting group. Marked variation from these values has been observed in disease and under various types of therapy; these changes will be described elsewhere. It is interesting to note, however, that in a group of unselected diabetic patients the concentration of glutathione is low but this lowered value of glutathione is compensated for by an increase in the concentration of γ -glutamylcysteine. Since the concentration of cysteinylglycine is considerably lowered in fasting patients, it is to be expected that values for the normal human will be found to be lower in the fasting state. Our data from a large group of patients would indicate that the level of glutathione is higher in the blood of the fasting than in the blood of the non-fasting human.

TABLE I
Glutathione in Blood

Values are not corrected for differences in volume in red blood cells (hematocrit) and are expressed as mg. per cent of reduced glutathione.

	Cysteinyl- glycine	γ -Glutamyl- cysteine	Glutathione	
			Present method	Nitroprusside method
	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Normals (5), non-fasting.....	5.8 ± 2.3	1.9 ± 0.5	16.9 ± 1.2	17.5 ± 1.1
Diabetics (10), fasting	1.8 ± 0.4	9.8 ± 3.6	11.8 ± 2.3	

Since the reaction with nitroprusside is positive for all sulfhydryl compounds but not for disulfide, and since by our method there is no distinction between the reduced or oxidized forms, there is no reason to expect agreement between the two methods. As is apparent from Table I, good agreement is obtained with blood of the normal human; such agreement does not apply when pathological blood is examined.

DISCUSSION

The method for the determination of glutathione and certain products of its hydrolysis has permitted us to make detailed studies of the metabolism of glutathione in disease. The documentation of these changes has required considerable time and effort and, in any event, we cannot hope to study more than a fraction of the available material. It is hoped, therefore, that the presentation of our methods will permit others to make a more precise study of the rôle of glutathione in physiological and pathological processes.

SUMMARY

Methods have been developed whereby glutathione and the products of its hydrolysis (cysteine, cysteinylglycine, and γ -glutamylcysteine) may be determined in blood. The method is illustrated with certain values for blood of the non-fasting normal human.

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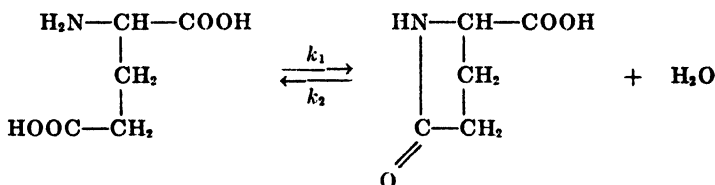
AN INTERPRETATION OF THE KINETICS OF GLUTAMIC ACID LACTAM FORMATION

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(Received for publication, March 24, 1950)

Examination of the admirable data of Wilson and Cannan (1) on lactam (pyrrolidonecarboxylic acid) formation from glutamic acid reveals that the variation with pH of the forward reaction rate at 100° can be related to the variation with pH of the population of the different ionic species present in aqueous solution, as has been done by Dusenbury and Powell (2) in the nitrous acid-ammonia reaction.



The concentrations of the ionic species shown in Table I, which are present in appreciable quantities, were calculated by the method of Hill (3), on the assumption of a total concentration ($\Sigma(A_i)$) of 0.05 M. Hill's equations simplify to the following relations.

$$(A_0) + (A_1) = \frac{[\Sigma(A_i)] \cdot [(H^+)^3 + K_1(H^+)^2]}{(H^+)^3 + K_1(H^+)^2 + K_1 \cdot K_{12}(H^+) + K_1 \cdot K_{12} \cdot K_{123}}$$

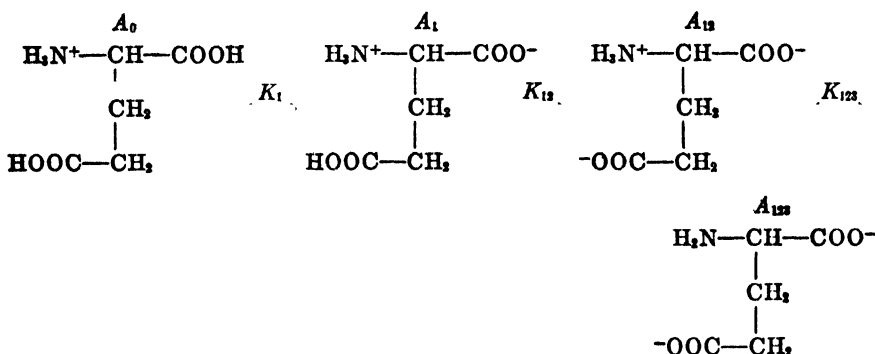
$$(A_{12}) = \frac{[\Sigma(A_i)] \cdot K_1 \cdot K_{12}(H^+)}{(H^+)^3 + K_1(H^+)^2 + K_1 \cdot K_{12}(H^+) + K_1 \cdot K_{12} \cdot K_{123}}$$

$$(A_{123}) = \frac{[\Sigma(A_i)] \cdot K_1 \cdot K_{12} \cdot K_{123}}{(H^+)^3 + K_1(H^+)^2 + K_1 \cdot K_{12}(H^+) + K_1 \cdot K_{12} \cdot K_{123}}$$

Hill's various K values (4) were corrected to 100° by the thermodynamic equation discussed by Pitzer (5). The approximate values for ΔC_p and ΔS , necessary in employing this equation, are estimated from those of similar acids measured by Harned and Owen (6). The ΔC_p and ΔS values and the corresponding acids are shown in Table I.

Fig. 1 shows the theoretical curves of $\log(A_i)/\Sigma(A_i)$ versus pH superimposed upon the experimental values for $\log k_1$ obtained by Wilson and Cannan. Coincidence requires the conclusion that A_0 , A_1 , and A_{123} are reacting species and that A_{12} is relatively non-reacting.

TABLE I
Ions and Equilibrium Constants



Equilibrium constant	pK ₂₅ ^a	pK ₁₀₀ ^a	ΔS ₂₅ ^a	ΔC _{p25} ^a	Analogous acid
K ₁	2.148	2.27	-8.1	-35.3	Alanine (K _A)
K ₁₂	4.31	4.69	-22.9	-41.8	Propionic acid
K ₁₂₃	9.96	8.46	-9.0*	-20.0†	Z [±]

* Z[±] + OH⁻ ⇌ ZOH⁻ ΔS = 10 (alanine, K_B)

H₂O ⇌ H⁺ + OH⁻ ΔS = -18.8

Z[±] + H₂O ⇌ H⁺ + ZOH⁻ ΔS = -8.8 ≅ -9.0

† See Harned and Owen (6), p. 52.

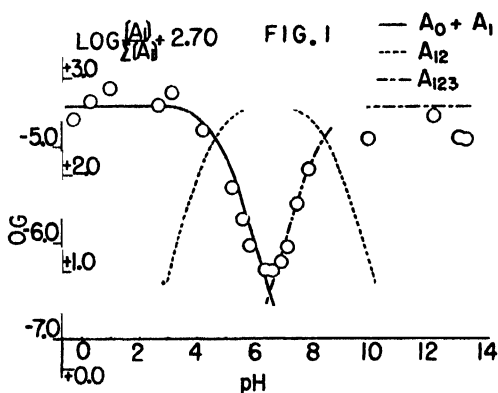


FIG. 1. Theoretical curves of $\log (A_i) / \Sigma(A_i)$ versus pH superimposed upon the experimental values for $\log k_1$.

It is seen that A_0 and A_1 have a free acid group and that A_{123} has a free amino group, whereas A_{12} has neither. One might surmise that, if the molecule could have existed in aqueous solution with both functional groups

free, a very rapid forward rate might have been observed. Interesting confirmation of this idea is supplied by the observation of Neuberger (7), in measuring the apparent K of glutamic acid esters potentiometrically at 25°, that, while the α -ethyl ester appeared to be perfectly stable in alkaline solution, the γ -ethyl ester and the diethyl ester were unstable, causing a steady drift in the electromotive force of the cell. It may be presumed that, in the absence of carboxylate ion resonance, the ester group has approached the free acid in reactivity, and lactam formation is proceeding apace.

SUMMARY

The rate of formation of glutamic acid lactam from glutamic acid in aqueous solution is shown to depend directly on the concentration of certain ionic species of glutamic acid in the solution.

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STUDIES ON THE INCORPORATION OF INJECTED CYTOCHROME *c* INTO TISSUE CELLS

II. INJECTION OF RADIOACTIVE CYTOCHROME *c* INTO NORMAL RATS*

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Considerable experimentation on the effects of cytochrome *c* administration preceded information on the metabolic fate of both injected cytochrome and that naturally found in the body (*cf.* (2)).

In a previous communication (3) the natural radioactive cytochrome in heart, kidney, and muscle of rats which had been given radioiron was not found to be diluted by injected non-radioactive cytochrome. This paper deals with the metabolic fate of injected radioactive cytochrome in so far as it can be traced by following its constituent iron in tissues, tissue fractions, and excreta.

The prosthetic group of the cytochrome molecule, in contrast to that of related compounds, cannot be reversibly liberated (4) and the iron is firmly bound in that group. Therefore, any specific use of iron-free fragments of the cytochrome molecule seems highly improbable. Although eventually liberated iron may be incorporated secondarily into proteins such as cytochrome, the turnover of endogenous cytochrome is of a negligible order, if the short experimental periods involved are considered.

EXPERIMENTAL

Biosynthesis of Iron-Labeled Cytochrome c—Several litters of rats, totaling 70 young, were kept in iron-free cages. Mothers and young were given access to a vitamin-supplemented milk diet only, beginning a few days after birth and continuing, after weaning, for the duration of the experiment. The procedure then followed is best illustrated by giving a representative protocol (Table I) on one of the rats (Rat L₂).

* A preliminary report of this paper has appeared (1). The radioiron used in this investigation was supplied by the Carbide and Carbon Chemicals Corporation, Oak Ridge, Tennessee, on allocation from the Isotopes Division, Atomic Energy Commission.

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A total of 9 mg. of Fe^* was injected into Rat L_2 , corresponding to about 55×10^5 c.p.m.; approximately 37×10^5 c.p.m., or 67 per cent, were found in the red cells and 28×10^5 c.p.m., or 0.5 per cent, in cytochrome. 75 per cent of the red cell iron and 80 per cent of the cytochrome iron were derived from the injected radioiron. Similar data for other rats of this series have been reported previously (3, 5).

The total amount of radioiron used for the biosynthesis of cytochrome was 1 gm. with a radioactivity of 1 mc. and 0.24 gm. with a radioactivity of 3 mc. With preparations of higher specific activity, which are now

TABLE I
Procedure for Biosynthesis of Radioactive Cytochrome c in Rats
Rat L_2 , born May 24, 1948.

Treatment	Date	Body weight	Hemoglobin
		gm.	gm. per 100 ml.
Bled	June 10	22	
"	" 14	25.5	10.3
"	" 15		
"	" 17	28	6.8
"	" 18		
" 0.5 mg. Fe^* intra-peritoneally	" 21	30	4.7
0.5 mg. Fe^* subcutaneously	" 23	30	
0.5 mg. Fe^* subcutaneously 3 times weekly	" 24-July 12		
0.15 mg. Fe^* intraperitoneally	July 13	84	13.1
0.15 mg. Fe^* intraperitoneally 3 times weekly	" 14-Sept. 23		
0.15 mg. Fe^* subcutaneously	Sept. 24	226	
Killed	Oct. 6	236	15.0

available, the production of anemia in the animals before the period of rapid growth may not be as critical for success as it was in the present work. The radioiron was alternately injected intraperitoneally and subcutaneously. An iron-ammonium citrate solution, made according to Peacock *et al.* (6), was not well tolerated by the rats in the amounts given. If ammonium was replaced by sodium in the same solution, no harmful effects were seen.

Isolation, Purification, and Analysis of Radioactive Cytochrome—When the rats weighed about 220 gm. (male) or 180 gm. (female), they were decapitated. Muscle and heart were minced and ground with sand.

Cytochrome *c* was isolated essentially according to the method of Keilin and Hartree (7). This procedure, with rat muscle, yields about 2.5 mg. of cytochrome per 100 gm. of fresh muscle, with a purity of 6 to 7 per cent based on protein dry weight and spectrophotometric data. The spectral purity, however, is satisfactory (*cf.* (5)), and non-cytochrome iron present is less than 3 per cent of cytochrome iron. The combined cytochrome solutions (500 ml.) thus obtained were diluted to twice their volume by an ammonium sulfate solution containing 0.5 gm. of the salt in 1 ml. The pH was adjusted to about 2. The turbid solution was then kept at 56° for 15 minutes. A heavy, almost white, precipitate was centrifuged after cooling and a reddish brown cytochrome was precipitated from the supernatant by trichloroacetic acid. This precipitate was dissolved in a few ml. of water by the aid of a few drops of NaOH. After dialysis against an 0.85 per cent NaCl solution and centrifugation, the solution was passed through a Swinny filter adapter with a Seitz filter¹ into a sterile bottle. About 11 ml. of a solution containing 4.6 mg. per ml. of cytochrome were obtained, which had a purity of 73 per cent based on protein dry weight and spectrophotometric data. The spectral purity was found in agreement with values reported in the literature for pure cytochrome (8), and non-cytochrome iron was less than 2.5 per cent of cytochrome iron. The biological activity in the succinoxidase test was high. The radioactivity was 2340 c.p.m. per mg. of cytochrome with the most efficient Geiger tube.

Injection of Rats with Radioactive Cytochrome and Exposure to Hypoxia—Seven white male rats were injected with varying amounts of the preparation, under slight ether anesthesia, through an exposed tail vein. Immediately after injection, Rats 1 and 3 to 6 were placed in a decompression chamber and decompressed to a simulated altitude of 20,000 feet within 30 minutes. The individual rats were kept there for different periods of time, as indicated in Table II. With Rat 5 the altitude was increased to 30,000 feet for 12 hours. Rat 1 was killed 45 minutes after injection by acute anoxia at an altitude of 40,000 feet. Rat 2 was placed in a breathing chamber which permitted withdrawal of blood from the tail and collection of the urine. A 10 per cent mixture of oxygen with nitrogen was passed through this chamber. Rat 7 was totally nephrectomized 2.5 hours before the injection of cytochrome and was not decompressed.

Preparation and Analysis of Organs and Excreta—Immediately before decapitation, blood was drawn from the animals by heart puncture. The red cells were separated and washed. The urine was collected in fractions in some of the animals. It was then dialyzed, cytochrome was

¹ Suggestion of Dr. R. B. Mitchell of this institute.

TABLE II
Recovery of Radioactivity after Injection of Iron-Labeled Cytochrome into Rats Exposed to Hypoxia

(a) organ analyzed; (b) per cent of dose recovered*; (c) per cent of radioactive cytochrome in cytochrome isolated.†

Rat No. Body weight, gm. Cytochrome injected, mg. Killed after injection, hrs.	1†		2		3		4		5		6		7‡	
	(b)	(c)	(b)	(c)	(b)	(c)	(b)	(c)	(b)	(c)	(b)	(c)	(b)	(c)
Kidney, total	28.1		35.5		36.6		35.2		35.9		31.8			
Cytochrome	11.3	54.4	1.9	11.6	0.10	1.54	0.56	3.0	0.18	2.2	0.42	3.4		
Ferritin, a.s.¶	0.66						5.8		3.0		8.4			
" Cd	0.31						2.2		1.25		3.1			
Liver, total	6.21		4.5		12.1		5.9		4.0		5.3		11.1	
Cytochrome	0.47	3.7	0.09	0.46	0.12	0.38	<0.03	<0.2	<0.01	<0.1	0.03	0.14	<0.11	0.21
Ferritin, a.s.¶	1.0		1.6		8.3		3.9		2.75		3.2		4.7	
" Cd	0.64		1.0				2.8		1.91		2.8		3.7	
Heart, total	0.08		0.05		0.12		0.15		0.05		0.01		0.16	
Cytochrome	0.02	0.41	<0.01	<0.14	<0.03	<0.3	<0.03	<0.5	<0.01	<0.12	<0.01	<0.12	0.06	0.48
Muscle, total			4.2		9.3		4.7		2.5		3.3		13.1	
Cytochrome			2.3	1.27	<0.03	<0.16	<0.03	<0.06	0.1	0.1	<0.04	<0.02	1.0	0.22
Brain	0.04		0.01		<0.03		<0.02		<0.01		0.02		<0.02	
Spleen	0.41		0.23		1.0		0.50		0.14		0.32		1.27	
Lung			0.17		0.18		0.14		0.15		0.15		0.34	
Glands¶							0.29		0.10		0.20		0.67	
Sex organs							0.38		0.25		0.59		1.69	
Pancreas					0.19				0.10		0.17		0.46	
Stomach					0.20		0.17		0.07		0.09		0.47	
Intestines, small					1.51		0.36		0.55		0.30		1.5	
" large							1.26		0.39		0.59		2.0	
Intestinal content					0.56		0.50		1.9		0.24		0.82	
Abdominal cysts							0.49							
Bones			2.0		2.6		1.7		2.6		1.37		6.7	
Skin							4.7		4.1		3.2		27.8	

Blood, total.....	4.4	1.9	2.6	2.6	1.83	5.8	3.6
Red cells.....	<0.06	<0.06		2.4	1.48	5.7	2.45
Plasma.....	4.4	1.9		0.2	0.35	0.07	1.15
Feces.....			1.8	1.5		2.58	1.35
Urine.....	30.5	27.2	18.4	39.4	45.6	43.5	
Total recovery, corrected*	69.7	75.8	87.1	99.9	100.2	99.6	73.0
Uncorrected*	63.7	69.5	80.0	93.5	96.0	93.2	64.3

* The values are corrected for the losses which could be defined and are calculated for the total respective organ.

† Columns c give the percentage of the injected radioactive cytochrome which is maximally present in the cytochrome isolated if contamination by radioactive breakdown products is neglected.

‡ Rat 1, died in acute anoxia at 40,000 feet.

§ Rat 7, bilaterally nephrectomized.

|| Ammonium sulfate precipitate; Cd:CdSO₄ precipitate of ammonium sulfate-precipitated fraction.

¶ Thyroid, thymus, and adrenals; the radioactivity in these glands was decreasing in the order used.

isolated, and all fractions were ashed separately. Contamination of feces by urine was not entirely excluded, except of course in the nephrectomized Rat 7. All parts of the body were freed from blood clots, washed, blotted, and kept frozen until analysis was started. The bones were freed of flesh and ashed as a whole. The skin around the site of injection and the tail were discarded. Skin and fur were well washed with soap. The digestive tract was emptied and washed.

Cytochrome was isolated from those organs, except brain, from which reasonable amounts can be isolated. The method of Rosenthal and Drabkin (8) was used for heart, kidney, and liver. Occasionally we used Carruthers' alumina adsorption (9) for final purification.² The latter method was used also for urine. For muscle, essentially the method described above for preparation and purification of radioactive cytochrome was used. In this case, emphasis was placed on purity of the isolated cytochrome rather than on quantitative isolation.

Ferritin was isolated from liver and kidney according to the method of Granick (10). To the dialyzed ammonium sulfate precipitate, CdSO_4 was added to a concentration of 4 per cent. No attempts were made to crystallize ferritin, as yields of crystalline ferritin from rat kidney and liver are reportedly poor (10) and quantitative isolation of the total ferritin fraction was intended. Radioactive cytochrome added *in vitro* before fractionation for ferritin did not appear in the ferritin fraction but in the residue and supernatant.

All fractions obtained from these analytical procedures were ashed separately; otherwise the whole organs were ashed, following essentially the procedures of Peacock *et al.* (6). Iron was precipitated as ferric hydroxide from smaller samples and as ferrous sulfide from samples which were of larger volume or contained trichloroacetic acid. In some cases (feces, intestinal content) extraction by the method of Vosburgh *et al.* (11) was necessary to eliminate salts which would interfere with subsequent electroplating. Sizable samples, such as muscle, bone, and skin, were treated after the dry ashing method of Vosburgh *et al.* (11). Plating was carried out as described before (3). Counts were taken with argon-filled Geiger tubes.³ In the earlier phase of the work these tubes had beryllium windows.

Discussion of Errors

The probable error in counting the final samples was kept well below the errors of sampling and of the analytical procedures involved in all

² The proper type of aluminum oxide was kindly supplied by Dr. C. Carruthers.

³ Thanks are due to Dr. L. M. Sharpe of Brookhaven National Laboratory for temporary lease of a Geiger tube.

those samples that contained more than 1 per cent of the totally administered radioactivity (probable error $< \pm 2.5$ per cent). In samples containing less than 1 per cent of the total activity, a probable error between 3 and 12 per cent was allowed, depending on the importance of the sample for the problem at hand. Standard samples of the stock cytochrome solution were analyzed for cytochrome and radioactivity at intervals during the work. Since the Fe^{59} activity had practically vanished, this procedure took sufficient account of the slow decay of Fe^{55} .

The greatest error is certainly introduced by the uncertainty of the volume actually injected. The error of about 0.05 ml., however, will affect only the total recovery, especially with the smaller injected doses, but hardly the pattern of relative distribution. Blood and plasma volumes were not determined but estimated from the values given by Wang and Hegsted (12). These values are considerably at variance with those reported by Berlin *et al.* (13). Using these latter values would reduce the activity calculated for blood and its fractions by a third, as well as the values given above for the radioactivity incorporated into the red cells of the animals used for biosynthesis. In some cases, however, it was evident from the amount of blood available by heart puncture that the blood volume exceeded the values given by the latter authors.

Blood which remained in the organs could generally not cause a serious error after heart puncture and decapitation. The values for tissues of large mass (muscle) or for those which take up only minimal radioactivity (brain, heart) may be somewhat elevated by trapped blood. Soon after injection, when intact radioactive cytochrome is still in the blood stream, the radioactivity in the cytochrome fractions may be augmented for the same reason.

RESULTS AND DISCUSSION

The analytical results on the distribution of radioiron after injection of labeled cytochrome are summarized in Table II. The concentration of cytochrome in the blood dropped rapidly. In Rat 2, 6.0, 3.2, and 1.9 per cent of the injected dose were found in the plasma after 30, 90, and 160 minutes, respectively. The period when considerable amounts of evidently intact cytochrome were present in the plasma coincided with the period of appearance of cytochrome in the urine. No radioactivity was found in the red cells at that time. Whereas the radioactivity in plasma declined to less than 0.1 per cent after 72 hours, the red cells had then taken up almost 6 per cent of the injected iron. Plasma radioactivity can no longer be considered as due to unchanged cytochrome at those later times.

The bulk of the injected cytochrome was removed by the kidneys,

which handle up to 80 per cent of a 5 to 10 mg. dose (Table II). About half this amount was spilled in the urine, most of this within 30 minutes after injection, and practically all within 2 hours. Minimal radioactivity appeared in later urine fractions. All the radioactivity present in the urine could be accounted for by the cytochrome found spectrophotometrically, so that no breakdown products seem to be involved. The radioactivity which passed in the outside fluid during dialysis of the urine was negligible.

Only in the rats which were killed 45 minutes and 3 hours, respectively, after injection at least part of the radioactivity which remained in the kidneys was associated with the cytochrome isolated. This is in agreement with results obtained by the usual analytical techniques (3, 14).

TABLE III

Distribution of Radioactivity in Kidney and Liver Fractions Obtained by Analyses for Cytochrome and Ferritin in Rat 5

Recovery in counts per minute actually obtained in the respective fractions. The dilution ratio in the cytochrome analysis was 1:5 for kidney and 1:3 for liver.

Fraction	Kidney, 0.75 gm.	Liver, 6.17 gm.	Fraction	Kidney, 0.72 gm.	Liver, 6.17 gm.
Cytochrome	12	1	Ferritin, CdSO ₄ ppt.	116	292
Supernatant	234	97	“ supernatant from CdSO ₄ ppt.	168	127
Ammonium sulfate ppt. at 56°	47	7	Supernatant from ammonium sulfate ppt.	158	76
Residue	3440	571	Residue	3105	158

Very small amounts of radioactivity, probably due to contamination from the other highly active fractions of the kidney, were found associated with cytochrome at later times. An example of the distribution of radioactivity in the fractions obtained by analysis of kidney and liver for cytochrome according to Rosenthal and Drabkin (8) and for ferritin according to Granick (10) is shown in Table III. Even if the radioactivity in the cytochrome fraction is considered to be due to real admixture of injected to endogenous cytochrome, the percentage of admixture after 24 hours hardly ever exceeded 3 per cent (Table II, Column *c*), which would be of no physiological importance.

The radioactivity in the ferritin fraction increased with the time after injection. After 45 minutes, less than one-fortieth of the radioactivity in the kidney was found in ferritin, after 72 hours more than one-fourth.

The liver removed almost constantly between 4 and 6 per cent of the

dose. Of this material only very small amounts were found to be associated with the isolated cytochrome even after 45 minutes or 3 hours, and minimal amounts after 24 hours. Increasing amounts were again found in ferritin, representing about two-thirds of the total in liver.

Of considerable interest in relation to therapeutic trials is the fact that only negligible radioactivity was found in brain, even in that animal which died in acute anoxia 45 minutes after injection. The same fact pertains to heart.

The bulk of endogenous cytochrome is in the skeletal muscle. The cytochrome isolated from the muscles of the injected animals contained minimal radioactivity, whereas the muscle residue contained an amount approaching that of liver. A similarly surprising fact was the considerable radioactivity found in the skin. Bone marrow was an expected site of radioactivity.

Little reference has so far been made to Rat 7. In this animal the main route of disposal was blocked by nephrectomy, all its organs seeming to take a higher load of the injected material. Surprising again was the high load found in muscle, even exceeding that of liver, and rather unexpected was the fact that the skin was the main site of disposal, taking up as much as 28 per cent of the dose.⁴ This finding in the nephrectomized animal is considered proof that the radioactivity found in the skin of the other animals was not due to contamination by urine. It is of interest to note that Rat 3, which for some reason did not excrete as much of the injected material in the urine, obviously approached the nephrectomized pattern.

The established distribution of the radioiron of the injected cytochrome leaves no doubt as to the fact that the injected material, although of the same species in this case, cannot be utilized by the body concurrently with its endogenous cytochrome. It is eliminated very rapidly by excretion via the kidneys and by breakdown. The most obvious sign of the latter is the early appearance of radioiron in liver and kidney ferritin and in the red cells. The organs around which most clinical interest centered, brain and heart, are found especially poor in dealing with injected cytochrome. This is found even in acute anoxia and at the peak of cytochrome concentration in plasma after injection.

A 10 mg. dose for a rat would compare to a 3 gm. dose for a human, an amount which has hardly ever been used in humans. The quantitative pattern of disposal of injected cytochrome in the human may certainly differ somewhat from that found in the rat; the fact that little, if any, is

⁴ When radioactive iron citrate containing the same amount of iron as the cytochrome injected into Rat 7 was injected into nephrectomized rats, about 1 per cent of the administered radioactivity was recovered from the skin.

utilized; however, is primarily a problem of permeability and, therefore, can be assumed to be common for all mammals.

SUMMARY

Iron-labeled cytochrome with a radioactivity suitable for tracer work has been produced by biosynthesis in rats which were made anemic and then injected with radioiron during their period of rapid growth. This cytochrome was injected into rats and the distribution of radioiron was established at various times after injection and exposure to hypoxia. Most of the injected material is rapidly disposed of by the kidneys by excretion or breakdown and the liberated iron appears early in ferritin and red cells. No evidence was obtained that the animal body can incorporate and utilize extraneous cytochrome as such. Brain and heart are among the organs containing the smallest amount of the injected material. In the nephrectomized rat the skin is the main site of disposal of injected cytochrome, followed by muscle and liver.

Thanks are due to Dr. H. Maier-Leibnitz for his invaluable advice in the early phase of the experiment and to Dr. K. R. Reissmann for help with care of animals and surgery. The interest and support of this work by Dr. H. I. Chinn, Lieutenant-Colonel R. B. Lewis, Lieutenant-Colonel J. M. Talbot, Colonel D. D. Flickinger, Major J. E. Pickering, and Captain G. A. Saxton, Jr., are gratefully acknowledged.

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α -HYDROXYTRYPTOPHAN, NOT AN INTERMEDIATE BETWEEN TRYPTOPHAN AND KYNURENINE

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Since the discovery of kynurenine by Kotake and Masayama (1), there have been extensive studies of the metabolism of tryptophan. Kynurenine is now regarded as an intermediate in the degradation of tryptophan by animal cells (1) and microorganisms (2). In addition there is evidence that kynurenine is a controlling factor in heredity (3).

However, very little is known about the process by which tryptophan is converted into kynurenine except for some limited enzymatic investigations (1) and indirect genetic experiments (3). Recently Mehler and Knox (4) have shown that *N*-formylkynurenine is enzymatically converted to kynurenine in a rat liver preparation.

Kotake, in his studies of tryptophan metabolism, postulated α -hydroxytryptophan to be a possible intermediate between tryptophan and kynurenine. Butenandt obtained natural α -hydroxytryptophan from H. Wieland and found it to be active, like kynurenine, in his experiments designed to control hereditary characters of *Drosophila*. At the same time, he admitted that, quantitatively, the former is less active than the latter and stated "Wir haben gefunden, dass *l*- α -Oxytryptophan qualitativ die gleichen physiologischen Wirkung entfaltet wie Kynurenin...In quantitativer Hinsicht ist es schwächer wirksam."

Recently, Suda, Hayaishi, and Oda (2) used the adaptive behavior of a *Pseudomonas* sp. to analyze the metabolic pathways of tryptophan, and their evidence suggests the following sequence of reactions: tryptophan \rightarrow kynurenine \rightarrow anthranilic acid \rightarrow catechol \rightarrow muconic acid.

Independently, Stanier and Tsuchida (5) working with a different strain of *Pseudomonas* sp. arrived at the following formulation: tryptophan \rightarrow kynurenine \rightarrow kynurenic acid.

Lately one of us (T. S.) (6) has succeeded in obtaining α -hydroxy-DL-tryptophan, and we have applied the method of "successive adaptation" to test Kotake's hypothesis that this substance is an intermediate between tryptophan and kynurenine.

EXPERIMENTAL

Behavior of Tryptophan-Adapted Cells and Non-Adapted Cells to α -Hydroxy-DL-tryptophan—For the details of the principle and the methods of the so called "successive adaptation" the reader is referred to the original article (2).

The Iizaka strain of *Pseudomonas* sp. was used throughout the experiments. So called unadapted cells (*i.e.*, cells not adapted to any compounds related to tryptophan) were grown in acid-hydrolyzed meat peptone medium (ordinary broth), the free tryptophan concentration of which is very low. To prepare the adapted cells, the specific substance, for instance tryptophan in this case, was added to this basal medium at a concentration of 0.2 per cent. The cells were harvested after 16 hours of incubation at 31–33°, washed twice with distilled water, and suspended in $m/15$ phosphate buffer of pH 7.4.

TABLE I
Oxygen Consumption of Adapted Cells

Cell suspension, ml. Phosphate buffer, $m/15$, pH 7.4, ml Substrate, 0.2 ml.	1.0 1.0	1.0 0.8 L-Tryptophan, 0.005 M	1.0 0.8 L-Kynurenine, 0.005 M	1.0 0.8 Hydroxy-DL- tryptophan, 0.01 M
Total oxygen consumption, c.mm.	80.4	246.5	203.0	81.2
Atoms of oxygen per mole of substrate.		14.8	11.8	0

Conditions, 30°, in air; 0.2 ml. of 10 per cent KOH in the central well

All experiments were carried out in the Warburg respirometer, at a temperature of 30° in an atmosphere of air. All the substrates were neutralized.

As shown in Fig. 1, tryptophan-adapted cells show no marked enzymatic activity towards hydroxytryptophan, whereas they are active with tryptophan and kynurenine. When limited amounts of the substrates are oxidized, adapted cells consume approximately 7.5 moles (15 atoms) of oxygen per mole of tryptophan and 6.0 moles (12 atoms) of oxygen per mole of kynurenine (Table I).

Presence of Inhibitor—As stated in previous reports, the enzymatic activity is very sensitive to small amounts of metal ions such as Ag^+ and Cu^{++} . During the preparation of hydroxytryptophan, stannous chloride was used and the latter had to be excluded as a possible inhibitor.

Double arm vessels were used and tryptophan (0.2 ml. of a 0.005 M solution) and hydroxytryptophan (0.2 ml. of a 0.01 M solution) were tipped into the main compartment at the same time independently. The oxygen consumption curve plotted against the time is just the same as the

curve with tryptophan shown in Fig. 1 and one can conclude that no inhibitory substance was present in the hydroxytryptophan preparation.

Adaptation to Hydroxytryptophan—To the ordinary broth as specified above, sterilized hydroxytryptophan was added to a final concentration of 0.2 per cent. Hydroxytryptophan was sterilized by filtration. The activity of the cells was then tested with tryptophan, kynurenine, and hydroxytryptophan.

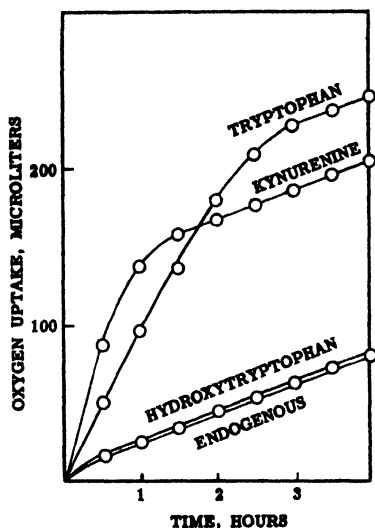


FIG. 1

FIG. 1. The oxidation of L-tryptophan ($1 \mu\text{M}$), L-kynurenine ($1 \mu\text{M}$), and α -hydroxy-DL-tryptophan ($2 \mu\text{M}$) by *Pseudomonas* sp. adapted to L-tryptophan. The amount of bacterial cells was determined by turbidimetric measurement to give the same concentration.

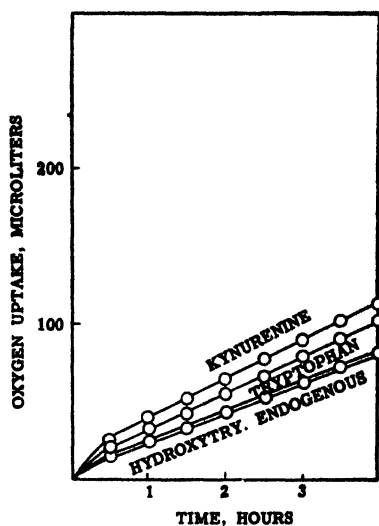


FIG. 2

FIG. 2. The oxidation of L-tryptophan ($1 \mu\text{M}$), L-kynurenine ($1 \mu\text{M}$), and α -hydroxy-DL-tryptophan ($2 \mu\text{M}$) by *Pseudomonas* sp. which is grown on ordinary broth without adding excess tryptophan. The amount of bacterial cells was determined by turbidimetric measurement to give the same concentration.

Hydroxytryptophan was found not to be metabolized at all, and the oxygen consumption for tryptophan and kynurenine was quite similar to that of non-adapted cells, as shown in Fig. 2.

DISCUSSION

The data obtained show that tryptophan takes up 3 atoms of oxygen more per mole of substrate than kynurenine and therefore indicates the possibility of oxidative conversion of tryptophan to kynurenine. However, synthesized hydroxytryptophan, which was postulated as a possible

intermediate by Kotake and by Butenandt, does not seem to be an intermediate between tryptophan and kynurenine in the case of this micro-organism.

The conversion of hydroxytryptophan to tryptophan or kynurenine is also doubtful in this case because hydroxytryptophan is not metabolized when the cells are grown in the presence of this substance.

SUMMARY

1. Hydroxytryptophan is not metabolized by tryptophan-adapted cells or by cells which were grown in the presence of hydroxytryptophan of *Pseudomonas*, whereas tryptophan and kynurenine are actively oxidized by the tryptophan-adapted cells.

2. It is most probable that α -hydroxytryptophan is not an intermediate between tryptophan and kynurenine in the case of *Pseudomonas* sp.

We wish to express our heartiest thanks to Professor David E. Green for his kind advice and aid in the preparation of this paper.

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KINETICS OF MALT α -AMYLASE ACTION*

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The action of α -amylase on undegraded starch and on dextrans of high molecular weight is roughly 100 times as rapid as its action on the smaller dextrans formed subsequently. The mechanism of this action has been studied in two ways. By kinetic methods, the course and rate of hydrolysis at varying substrate concentrations are determined. Thus Myrbäck and Johansson (1) calculated the affinities between the varying split-products and the enzyme from a study of the course of hydrolysis at different substrate concentrations. Similarly Bernfeld and Studer-Pécha (2) have investigated the rate of action of various α -amylases on amylopectin at varying substrate concentrations. Alternatively, the reaction can be stopped at different stages and the products characterized as to chain length, structure, and subsequent capacity for hydrolysis. Myrbäck and Lundén (3) have made a substantial contribution with this approach. More recent studies include that of Alfin and Caldwell (4) on the digestion products of pancreatic amylase action on potato starch and on corn amylose. Swanson has recently characterized the products of action of salivary amylase on glycogen and on amylose (5). Perhaps the current view of the whole case has been succinctly expressed by Myrbäck (6) that α -amylase "can attack substantially all normal glucosidic linkages in a chain molecule with velocities varying with the distance of the linkage in question from the end-groups."

Three phases of the action of crystallized malt α -amylase (7) are considered in this report: (a) the ultimate extent of the hydrolysis of starch, (b) the change in the velocity of hydrolysis with increasing substrate concentrations for a variety of substrates other than starch, and (c) inhibition by products of the reaction. It has been found that α -amylase will ultimately hydrolyze 50 per cent of the glucosidic bonds, with maltose, glucose, and apparently trisaccharides as end-products. Calculation according to classical enzyme kinetics indicates that, for all substrates with chains more than 10 glucose units long, the affinity constant K is invariant within experimental limits. The amyloses are hydrolyzed at about double the maximum rate of the amylopectins. Results of the inhibition studies

* Enzyme Research Division Contribution No. 126.

indicate that the end-products of α -amylase action inhibit non-competitively, whereas incompletely hydrolyzed split-products compete with unhydrolyzed substrate for the enzyme.

Materials and Methods

Initial Rate Studies—The substrates used in studying the initial rate are listed in Table I. The following samples of amylose were the same as those used by Potter and Hassid¹ (8): potato, tapioca, Easter lily, corn, and acid-modified corn amyloses. The amylopectin samples and wheat amylose were prepared by the thymol method of Haworth, Peat, and Sagrott (10). The glycogen sample was obtained from a commercial source. The β -limit dextrin was obtained by allowing crystalline β -amylase from sweet potatoes (11) to act on corn amylopectin until no further increase in reducing action was detected. The dextrans were precipitated from the digestion mixture by the addition of alcohol to 50 volumes per cent and then washed with alcohol and ether and dried.

The degree of polymerization was obtained by determining the reducing values of these substrates on dinitrosalicylic acid, as described below. For comparison, the degree of polymerization of the amyloses used is shown, as determined by Potter and Hassid (8, 9) from osmotic pressure measurements on the corresponding acetates. The average chain length determined by Potter and Hassid by means of periodic acid oxidation and the viscosities of these samples are also included in Table I.

It should be pointed out that the molecular weights of these substrates are still a matter of some disagreement. Thus Kerr and Cleveland (12) and Meyer *et al.* (13) have presented evidence that the molecular weights of the amylopectins are of the same order of magnitude as those of the amyloses.

For the determination of the initial velocities of hydrolysis, a 500 mg. sample of the substrate was dissolved in 40 ml. of 0.5 N NaOH. The pH was adjusted to 4.75 with 2 ml. of glacial acetic acid, and the volume of solution adjusted to 50 ml. Three different substrate concentrations were used. They contained 5.0, 2.5, and 1.25 ml. of the stock substrate solution respectively, 0.0, 2.5, and 3.75 ml. of buffer (prepared as for the stock substrate), 4.9 ml. of saturated calcium sulfate solution, and 0.1 ml. of a 1:100 dilution of stock enzyme solution to give a total of 10 ml. of digestion mixture. The stock enzyme was a solution of thrice crystallized malt α -amylase (7), containing 3.7 mg. of protein per ml. The final concentration of α -amylase per ml. of digestion mixture was calculated from the reported molecular weight of 59,500 (7) to be 6.2×10^{-6} μ M of enzyme

¹ The generous gift of these amylose samples from Professor W. Z. Hassid of the University of California is gratefully acknowledged.

per ml. The substrate concentrations were calculated as 30.8, 15.4, and 7.7 μM of glucose existing in glucosidic linkages per ml. of digestion mixture.²

Substrate hydrolysis at 30° was followed by determining at intervals the increase in the reducing action of 2 ml. of the digestion mixture on 1 ml. of dinitrosalicylic acid reagent³ (14). After boiling in a water bath for exactly 5 minutes, the reagent-sugar mixture was cooled, diluted to 10 ml. with distilled water, and its per cent transmission with a 540 $\text{m}\mu$ filter was read in an Evelyn photoelectric colorimeter. A more complete series

TABLE I
Substrates for α -Amylase Action

Substrates (amyloses)	Degree of polymerization from		Average chain length*	Intrinsic viscosities*
	Reducing values	Osmotic pressure*		
1. Potato.. . . .	1000	930	930	1.95
2. Easter lily.....	600	620	620	1.06
3. Tapioca.	1250	1,300	650	2.25
4. Corn	760	800	270	1.23
5. " (acid-treated).	400	400	130	
6. Wheat.				
7. Potato amylopectin.		(40,000)	15-25	
8. Corn "		(30,000-37,000)		1.3
9. Wheat "				
10. β -Limit dextrin.		(25,000)	8-10	
11. Potato starch, soluble	200			
12. α -Dextrin...	10-11			
13. Glycogen.. . . .		(40,000)	5-10	

* Samples 1 to 5 and data are those of Potter and Hassid (8, 9).

of concentration was run on one substrate, wheat amylose. Retrogradation was minimized by neutralizing each aliquot of the cold alkaline amylose solution within a few seconds before adding the enzyme.

Inhibition Studies—The substances used as inhibitors were (a) maltose hydrate, (b) the non-fermentable "limit dextrin" resulting from the combined action of α - and β -amylase on soluble starch (15), and (c) α -dextrin, obtained by precipitation with alcohol from a digest of soluble starch by

² This method of expressing substrate concentration has been found very convenient in the presentation of these results and is adhered to throughout the paper. It is regretted that no short term is available to express the idea behind the misnomer "moles of glucosidic bonds."

³ This sugar reagent contained 1.0 gm. of dinitrosalicylic acid and 30 gm. of Rochelle salt in 100 ml. of 0.6 N NaOH. The reaction was found to follow Beer's law according to the relation $M = 2.8L$, where M = the microequivalents of maltose and L = the optical density of the solution of the reduced reagent.

α -amylase at the point where about 10 to 12 per cent splitting of total glucosidic bonds had occurred.

As the inhibitors are also reducing substances, total reducing values would be prohibitively large in comparison with the increase owing to hydrolysis. Accordingly, the rate of dextrinization was determined by the color of the digest with iodine. The method is essentially that described by Schwimmer (16), except that varying concentrations of substrate were used. In the present case 5 ml. of enzyme solution (2.1 units or 28 γ of crystalline α -amylase) were added to 10 ml. of Lintner soluble starch at pH 4.75, containing also the amount of inhibitor indicated in Table IV. After incubation at 30° for various lengths of time, an aliquot of the digest was added to an iodine-potassium iodide mixture, the volume adjusted to 15.5 ml., and the color of the resulting dextrin-iodine complex read in an Evelyn photoelectric colorimeter at 660 $m\mu$. The aliquot was so taken that the amount of total carbohydrate added to the iodine-iodide solution was kept constant, i.e., 5.76 mg. per ml. of the original solution of Lintner soluble starch. Thus the highest starch concentration used was 11.52 mg. per ml. of digest, and 0.50 ml. of the digest was added to the iodine solution; when half as much starch was used, 1 ml. of digest was added to the iodine solution, and so forth. 1 unit of enzyme is defined as that amount in the digestion mixture which in 10 minutes decreases the reading in the colorimeter to 50 per cent transmission at 660 $m\mu$.

The relative rates of hydrolysis were then calculated from the apparent units obtained in this manner by dividing the latter by the appropriate dilution factor, since the units indicate the percentage change per unit of time rather than the actual amount of substrate changed. The rate at the highest starch concentration without inhibitor has been arbitrarily taken as 100. For example, when 11.52 mg. of substrate per ml. were used, the time required to obtain the standard color with 0.5 ml. of digestion mixture was 4.76 minutes, corresponding to 2.1 (10/4.76) units of enzyme; this velocity is thus equal to 100. When 5.76 mg. of substrate were used, 1 ml. of digest was added to the iodine-iodide solution and the time required to reach the same color value (after appropriate dilution) was 2.92 minutes. The apparent activity was therefore 3.42 units, the velocity was equal to one-half this value (1.71), and the relative velocity was 81.5.

Extent of Digestion—The extent of digestion was carried out in essentially the same manner as described before (7), the hypoiodite titration being used to determine the appearance of reducing sugars (17).

Graphic Analysis of Results—The classical dissociation constant of Michaelis and Menten (18) (K_s) for enzyme-substrate complex formation and the maximum velocity (V) obtainable for a given enzyme concentration are taken from the Lineweaver-Burk equation (19).

$$\frac{1}{v} = \frac{K_s}{V} \left(\frac{1}{S} \right) + \frac{1}{V} \quad (1)$$

or

$$v = \frac{VS}{K_s + S} \quad (1, a)$$

where v = the initial reaction rate and S = the substrate concentration. This equation is based upon the following assumptions: (1) the rate is proportional to the concentration of enzyme-substrate complex, and (2) the maximum rate is proportional to the total enzyme (e) present,

$$e = kV \quad (2)$$

where k , when expressed in molecular terms, is a measure of the turnover number, and (3) the concentration, S , is essentially unchanged by combination with the enzyme.

When an inhibitor in concentration I competes with substrate for the enzyme (20), then

$$\frac{v_0}{v_I} = 1 + \frac{K_s}{K_I} \frac{I}{K_s + S} \quad (3)$$

where v_0 is the velocity in the absence, v_I is the velocity in the presence of I at concentration S , and K_I is the dissociation constant for enzyme-inhibitor complex. When the inhibition is non-competitive,

$$\frac{v_0}{v_I} = 1 + \frac{I}{K_I} \quad (4)$$

Results

The most complete set of data was obtained with wheat amylose. Its hydrolysis at varying substrate concentrations as a function of time is shown in Fig. 1, and the initial rates of hydrolysis are listed in Table II. The variables $1/v$ and $1/S$ are plotted against each other in Fig. 2. The straight line was obtained by applying the method of least squares to the data. The absolute value of the x intercept is the reciprocal of the K_s value (4.8 microequivalents of glucosidic bonds per ml.); the value of the y intercept is the reciprocal of the V value for wheat amylose within experimental error (0.26 microequivalent of glucosidic bonds hydrolyzed per ml. per minute).

Data for the other substrates were subjected to the same treatment, with the limitation imposed upon them that the K_s values were all assumed to be equal to 4.8. Table III contains initial velocity for these substrates along with the V values obtained graphically (except for β -limit dextrin and glycogen). The curves for theoretical initial velocity *versus* substrate

concentration obtained for each substrate with these constants and Equation 1, α are shown in Fig. 3. With $K_s = 4.8$, these were the best fits

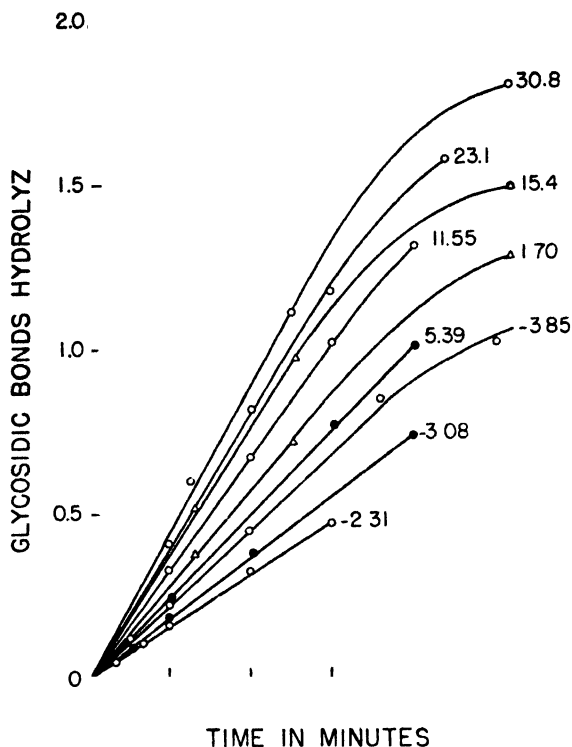


FIG. 1. The initial course of hydrolysis of wheat amylose by malt α -amylase at varying substrate concentrations; the initial concentration of substrate (expressed as microequivalent of glucose in glucosidic linkage per ml. of digestion mixture) is shown for each curve.

TABLE II
Initial Velocity of Hydrolysis of Wheat Amylose by Malt α -Amylase

S^*	v^*	S	v	S	v
30.8	0.232	11.55	0.187	3.85	0.115
23.1	0.214	7.70	0.158	3.08	0.099
15.4	0.206	5.39	0.135	2.31	0.086

* S and v are expressed in terms of microequivalents of bonds per ml. and microequivalents of bonds hydrolyzed per ml. per minute, respectively.

obtainable for the experimental data, except for the values obtained with the substrates β -limit dextrin and glycogen. With β -limit dextrin the

best curve was obtained when $K_s = 6.9 \mu\text{M}$ of glucosidic bonds per ml. and $V = 0.20 \mu\text{M}$ of bonds hydrolyzed per ml. per minute. For glycogen the corresponding values were $K_s = 20$ and $V = 0.14$.

The course and extent of hydrolysis of soluble starch at varying concentrations of α -amylase and in the presence of crystalline sweet potato β -amylase are shown in Fig. 4. As can be seen, the ultimate degree of hydrolysis never seems to have exceeded 50 per cent hydrolysis of the glucosidic bonds. However, qualitative tests (modified Barfoed test and osazone formation) indicated the presence of glucose in the hydrolysates. Hence probably some trisaccharide was also present to balance the excess over 50 per cent hydrolysis.

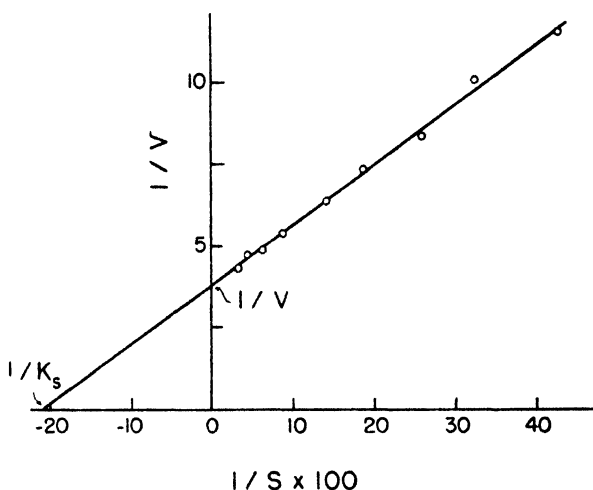


FIG. 2. Plot of $1/v$ against $1/S$ for wheat amylase

The relative rates of hydrolysis of soluble starch as functions of substrate and inhibitor concentration are shown in Table IV. A plot of the pertinent variables ($1/v_0$ against $1/S$) is presented in Fig. 5, *a*. The relation between the reciprocal of the fractional inhibition (v_0/v_i) and the inhibitor concentration for the α -dextrin (Fig. 5, *b*), maltose (Fig. 5, *c*), and limit dextrin (Fig. 5, *d*) reveals some rather interesting differences. Thus maltose seems to inhibit non-competitively over the whole range of substrate concentration, whereas the limit and α -dextrins exhibit considerable inhibitory action only at the lower substrate concentrations. At these lower concentrations, the limit dextrin seems to inhibit non-competitively, the α -dextrin competitively. Demonstration of this competition is shown by the constancy of the value for the inhibition constant K_i , when Equation 3 is applied. From the calculated value of $K_i = 2.77 \text{ mg. per ml. from}$

Fig. 5, *a*, the corresponding values for K_I at substrate concentrations equal to 1.44 and 2.88 mg. per ml. are 6.1 and 6.4 mg. per ml. of inhibitor respectively. At a substrate concentration of 5.76 mg. per ml. the corresponding value for K_I is equal to 12.5 mg. per ml., thus showing both the decreased inhibitory action and the indeterminate nature of the competition at a higher substrate concentration. Application of the equation for non-competitive inhibition (Equation 4) to the limit dextrin and to maltose

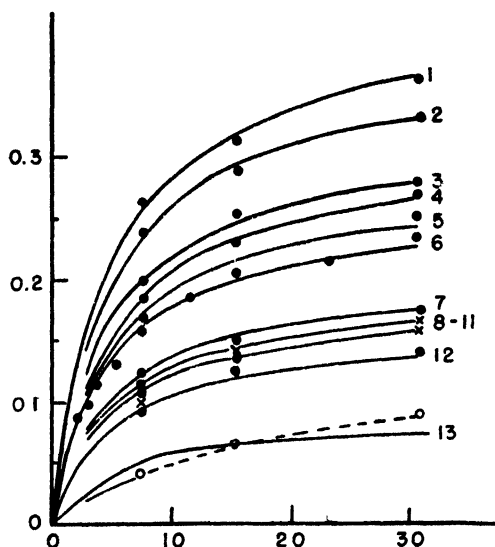


FIG. 3. Velocity (v) of hydrolysis of various substrates as a function of substrate concentration. The numbers in the graph identify the substrates as listed in Table I. The points (●) represent the experimentally determined values of v for all substrates except β -limit dextrin (×) and glycogen (○). The curves represent the calculated v values from Equation 1, *a*, with an assumed K_s value of 4.8 and V values as described in the text. ○ shows the calculated curve for glycogen with the values shown in Table III.

yields inhibition constants equal to 27 and 57 mg. per ml., respectively. Thus the inhibitory activity of these end-products increases in the order of maltose, limit dextrin, and α -dextrin.

DISCUSSION

The conclusion that the K_s values are constant for all but two of the substrates tested is based, of course, on the assumption that a measurable K_s exists for each substrate. Except for wheat amylose, the velocity of hydrolysis of these substrates was determined when the enzyme was

TABLE III
Constants for α -Amylase Action

Substrate	Initial velocities (v)			K_s	V	$k \times 10^{-4}$
	$S^* = 30.8$	$S = 15.4$	$S = 7.7$			
Potato	0.36	0.31	0.26	4.8	0.42	6.8
Easter lily	0.33	0.29	0.24	4.8	0.39	6.3
Tapioca	0.27	0.26	0.20	4.8	0.32	5.2
Corn	0.27	0.23	0.18	4.8	0.30	4.9
“ amylose acid-treated	0.26	0.21	0.17	4.8	0.23	4.6
Wheat amylose...	0.23	0.21	0.16	4.8	0.26	4.2
Potato amylopectin	0.17	0.15	0.12	4.8	0.20	3.3
Corn “	0.16	0.14	0.11	4.8	0.19	3.1
Wheat “	0.16	0.14	0.11	4.8	0.19	3.1
β -Limit dextrin	0.16	0.13	0.10	6.9	0.20	3.3
Lintner soluble starch	0.16	0.14	0.11	4.8	0.18	2.9
α -Dextrin	0.14	0.13	0.10	4.8	0.16	2.6
Glycogen	0.09	0.06	0.04	20.0	0.14	2.3

* S and K_s are expressed as microequivalents of bonds per ml., v and V as microequivalents of bonds hydrolyzed per ml. per minute, and k as microequivalents of bonds hydrolyzed per micromole of α -amylase per minute.

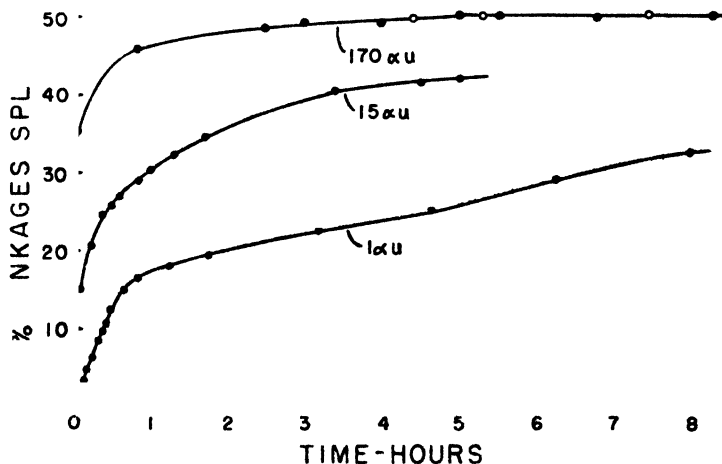


FIG. 4. Course and extent of hydrolysis of 2 per cent soluble starch by 1, 15, and 170 α -amylase units at 30°. \circ represents hydrolysis obtained with 170 units of α -amylase plus 10 units of sweet potato β -amylase

evidently approaching saturation with substrate (see Fig. 3). However, this assumption is not unreasonable, since it has been rigorously proved for wheat amylose, for which substrate concentrations in the region of the K_s values were used. Furthermore, the calculated values based on this

assumption fitted the experimental values fairly well. It should be pointed out that constancy of K_s on a gm. or equivalence basis actually means that K_s on a molecular basis (which presumably would have more significance with regard to mechanism) is related to K_s on a gm. basis as follows: K_s (molecular) = K_s (gm.)/(mol. wt.). Thus the affinity of the enzyme for substrate is directly proportional to the molecular weight. Obviously

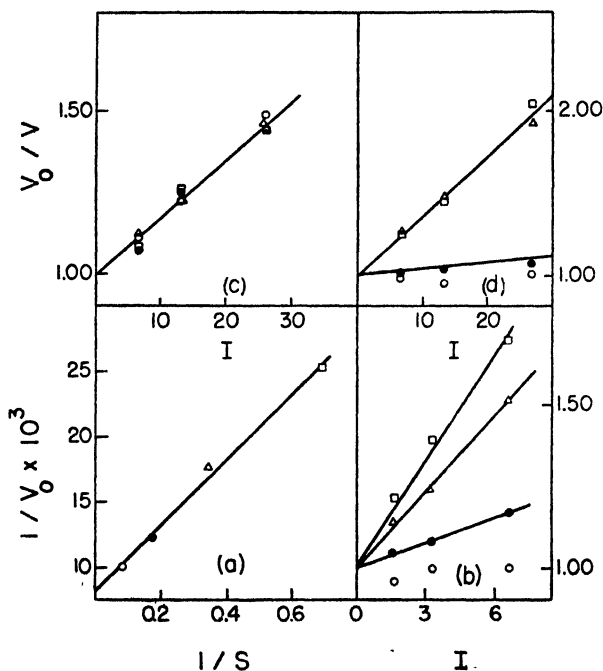


Fig. 5. Summary of the effect of inhibitor concentration (I) on the rate of dextrinization of soluble starch by malt α -amylase at varying substrate concentration (the symbols are defined in the text). The substrate concentrations are as follows: 11.52 (○), 5.76 (●), 2.88 (△), and 1.44 (□), mg. per ml. respectively.

the data have significance with respect to the above relation only if the substrates used do have different molecular weights.

The K_s value found for almost all the substrates is of the same order of magnitude as that found by Bernfeld and Studer-Pécha (2), using pancreatic and bacterial amylase acting on amylopectin, and that of Myrbäck and Johansson (1), using malt α -amylase acting on soluble starch. The apparent independence of the size and structure of the substrate exhibited by the K_s values indicates that the essential substrate for α -amylase is the "glucosidic" linkage rather than the whole molecule; i.e., one linkage is as susceptible as another to combination with enzyme, provided the

linkage is in a straight chain of at least 10 glucose units. Thus glycogen according to Meyer and Fuld (21) does not contain any chains greater than 7 units long. Whereas the "exterior" chains of amylopectin are 15 units long, the "interior" chains, the only ones present in β -limit dextrin, average about 7 to 8 glucose residues (13). This may explain why these two substrates have K_s values higher than the other substrates tested. The great decrease in velocity of hydrolysis of starch which occurs when the fragments average about 8 units and lower is also in accord with the concept that K_s values are independent of chain length and structure when above about 10 units.

On the other hand the V values seem to depend in some way on both the molecular weight and degree of branching. Thus the amyloses hydro-

TABLE IV
Effect of Digestion Products on Velocity of Dextrinization of Soluble Starch by Mall α -Amylase

Inhibitor	Inhibitor concentration, mg. per ml	Substrate concentration, mg. per ml.			
		11.32	5.76	2.88	1.44
		Relative hydrolysis velocities			
None	0.00	100	82	56	39
Maltose	6.35	90	77	50	36
	12.70	82	66	46	31
	25.40	67	57	38	27
	6.68	102	80	44	31
Limit dextrin	13.35	106	81	38	27
	26.70	100	84	29	19
	1.67	105	78	49	32
α -Dextrin	3.34	100	76	45	28
	6.68	100	70	37	23

lyze at about twice the rate of the amylopectins. If the differences in V within the amylose series are to be considered significant, then we see that the highest velocity is obtained with the least branched and most highly polymerized substrate (Table I). The reason for these differences is not immediately apparent.

It is of interest to note that the hydrolysis of wheat amylose followed a zero order reaction curve at very low substrate concentrations (Fig. 1). Simple enzyme-substrate combination predicts that the reaction should be first order at these concentrations, since the enzyme is not saturated with the initial substrate at these low concentrations. However, if one assumes that the enzyme recombines successively with smaller and smaller split-products, and with equal affinities for these split-products, then the enzyme

will be combined to at least as great a degree after time t as at zero time. The reaction will then follow zero order kinetics. This supposition is in complete agreement with the finding that the K_s values are constant for all substrates whose chain length is greater than about 10 glucose units. It also explains why the course of hydrolysis is always zero order to the point of appearance of large amounts of the smaller dextrans.

One would expect *a priori* that substances which can combine with α -amylase to form a hydrolyzable enzyme-substrate complex would compete with another substrate (*viz.* soluble starch) for active sites on the enzyme. This in fact was found for the inhibition with α -dextrin, which can be further hydrolyzed by the enzyme. On the other hand, unhydrolyzable or difficultly hydrolyzable end-products (for example maltose) were found to inhibit in a non-competitive manner.

There may be significance in the finding that the α -dextrin, whose K_s value falls within the same range as that of the majority of the substrates, inhibits competitively, whereas substances less than 10 glucose units long (maltose, "limit" dextrin) inhibit non-competitively.

Previous studies on the extent of hydrolysis of starch with α -amylase usually show that the "second" slow phase of hydrolysis has no definite end-point, but continues with lessening velocity until about 45 to 50 per cent of the linkages are split (22). We have definitely shown that by using a high enough concentration of enzyme the hydrolysis can proceed within a comparatively short time to 50 per cent, when it apparently stops. The implied presence of trisaccharides in the final hydrolysate may explain why no "isomaltose" is found in hydrolysates from malt amylase action (23). From this point of view at least, malt α -amylase differs from fungal amylase action (24).

SUMMARY

The initial rates of hydrolysis by crystalline malt α -amylase have been determined at varying concentrations of some amyloses, amylopectins, dextrans, and of glycogen. The inhibition of starch hydrolysis by products of the reaction has also been investigated. The amyloses were hydrolyzed at maximum rates which are about twice the maximum rates found for the amylopectins and glycogen. The highest maximum velocity (corresponding to a turnover number of 68,000) was obtained with amyloses which did not contain branch points.

Calculations, according to the classical Michaelis-Menten theory, revealed that, within experimental error, the affinities of almost all of the substrates for the enzyme showed a constant value on a "gm." or "equivalent" basis. On a molecular basis, the affinity of the enzyme for the substrate is directly proportional to the molecular weight. Only the "affinity"

constants for glycogen and probably β -limit dextrin were significantly lower than for the other substrates. It is suggested that lowered affinities are apparent only in substrates whose chain length is less than approximately 10 glucose units. This conclusion also agrees with the results of inhibition studies which indicate that the end-products of α -amylase action inhibit non-competitively, whereas incompletely hydrolyzed split-products compete with starch for the enzyme.

The ultimate extent of the hydrolysis of starch by malt α -amylase was found to be at 50 per cent splitting of the total glucosidic linkages. Maltose, glucose, and trisaccharides are the apparent end-products.

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THE UTILIZATION OF ALLOISOLEUCINE BY *LACTOBACILLUS ARABINOSUS**

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Smith and Greene (1) and more recently Horn *et al.* (2) have reported the occurrence of alloisoleucine in commercial samples of DL-isoleucine. This finding is of importance in connection with the selection of standards for microbiological assays. In the present authors' laboratory it has been found that a number of recrystallizations are necessary to free synthetic isoleucine completely from all traces of alloisoleucine, and it is even more difficult to obtain alloisoleucine completely free of isoleucine.

Hegsted (3) reported that L-alloisoleucine has 25 to 50 per cent of the activity of L-isoleucine for *Lactobacillus arabinosus*. The values obtained varied with the length of the growth period used in the tests.

In the present investigation it was found that the activity of alloisoleucine for *L. arabinosus* is dependent on the presence of pyridoxamine in the medium. It is the purpose of this report to present studies on the mechanism of alloisoleucine utilization and the relationship of vitamin B₆ to the process.

EXPERIMENTAL

Preliminary tests showed that microbiological assays for the isoleucine content of mixtures of alloisoleucine and isoleucine with *Leuconostoc mesenteroides* P-60, *Streptococcus faecalis* R, and *L. arabinosus* 17-5 all gave concordant results, provided that the medium for *L. arabinosus* did not contain pyridoxamine. No evidence was found that *S. faecalis* R or *L. mesenteroides* could utilize alloisoleucine under any of the conditions studied. Hence, microbiological assay with either of these two organisms provided a method for the determination of the isoleucine content of mixtures of the isomers.

The DL-isoleucine used in these experiments was prepared by the synthetic method described by Marvel (4). The activity of the preparation, as determined with *S. faecalis* R, slowly increased during five recrystallizations from 33 per cent ethanol, and remained constant through three additional recrystallizations. The final activity was 109.5 per cent of a

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commercial sample of DL-isoleucine which probably contained some allo-isoleucine.

Mixtures containing substantial amounts of DL-alloisoleucine were prepared by concentrating the mother liquor and collecting a second crop of crystals from the crude isoleucine filtrate obtained in the synthesis of isoleucine by the above method. The precipitate contained considerable amounts of ammonium bromide which were removed by precipitating the bromide ion with silver sulfate. A slight excess of silver was removed by careful addition of hydrochloric acid. Sulfate ions were removed with barium hydroxide, and ammonia by vacuum distillation. The crystals obtained by concentrating the solution to near saturation and adding 95 per cent ethanol to give a 33 per cent alcohol solution had a nitrogen content of 10.31 per cent (theoretical 10.61 per cent), indicating that the preparation consisted of isoleucine isomers. Assay with *S. faecalis* R showed a DL-isoleucine content of 63 per cent.

Preparations which contained higher percentages of alloisoleucine were obtained by a series of fractional recrystallizations from water. In each recrystallization the filtrate was higher in alloisoleucine than the crystals. The fractionation procedure, involving more than 50 recrystallizations and recombinations, yielded a product containing approximately 90 per cent DL-alloisoleucine and 10 per cent DL-isoleucine.

The isoleucine assays with *L. mesenteroides* were carried out by a modification of the procedure described by Dunn *et al.* (5). Procedures for the assays with *S. faecalis* R were as reported by Lyman *et al.* (6).

The composition of the medium used in tests with *L. arabinosus* was the same as that described by Kuiken *et al.* (7), except that tomato eluate was omitted and, when indicated, pyridoxamine was substituted for pyridoxine. The quantities of pyridoxine and pyridoxamine in these tests were 2 γ per 10 ml. of culture medium.

The amount of growth which took place in the test cultures was determined by titrating the acid produced with 0.1 N NaOH.

RESULTS AND DISCUSSION

Fig. 1 represents standard isoleucine growth curves with *L. arabinosus* in a medium containing pyridoxine and in a medium containing pyridoxamine. Only slightly better growth was obtained at the various levels of isoleucine as a result of adding pyridoxamine. In contrast to this behavior, when a mixture of isoleucine and alloisoleucine was used for the curve, the effect of adding pyridoxamine was quite striking (Fig. 2). The increased growth of the organism in the presence of this vitamin indicates substantial utilization of alloisoleucine.

The response of *L. arabinosus* to alloisoleucine in the presence of py-

ridoxamine at the lower growth levels was very slight, but increased rapidly as a function of the amount of mixed isomers added. Fig. 3 shows the results of an assay of a mixture of alloisoleucine and isoleucine in media with pyridoxine and with pyridoxamine. It will be noted that the apparent isoleucine content as represented by the top curve ranges from approximately 36 to 83 per cent as compared to a consistent value of 21.1 per cent obtained with the medium containing only pyridoxine. The

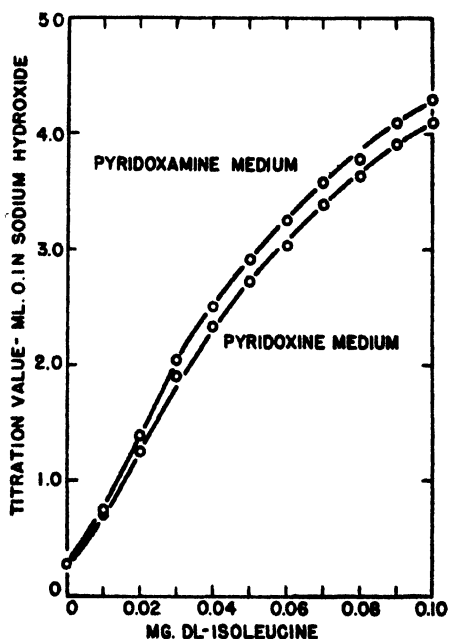


Fig. 1

FIG. 1. Standard isoleucine curves with *L. arabinosus*

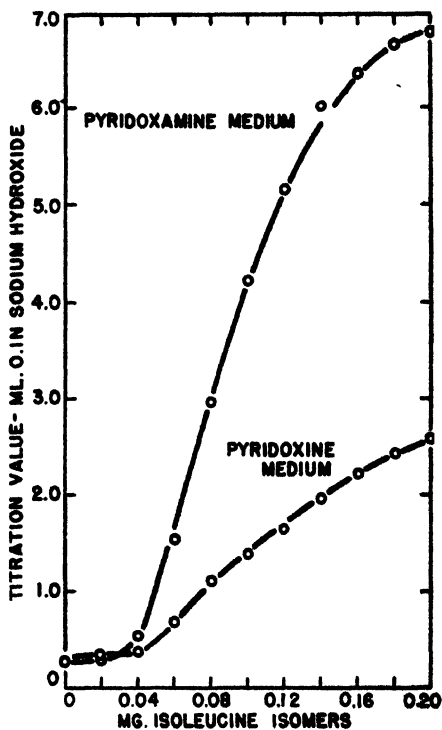


Fig. 2

FIG. 2. Growth response of *L. arabinosus* to a mixture of isoleucine isomers

value obtained with *L. arabinosus* in the pyridoxine medium was in good agreement with the values obtained with *S. faecalis* R (20.1 per cent), and with *L. mesenteroides* (19.5 per cent). The difference in activity of mixtures of alloisoleucine and isoleucine when assayed with *L. arabinosus* in a medium containing pyridoxamine, as compared to assays in a medium containing only pyridoxine, forms a basis of estimating the alloisoleucine content of mixtures of the isomers.

Experiments were then designed to determine whether alloisoleucine was

being utilized *per se*, or was being converted to isoleucine before incorporation into the cell constituents. *L. arabinosus* organisms were grown in a series of flasks, each containing 1 liter of medium, as shown in Fig. 4. The cultures were incubated for 3 days, and the cells were collected by centrifugation, washed, dried, and assayed for L-isoleucine with *L. arabinosus* by use of the two different media.

In Tests C and D, the apparent isoleucine content of the cells was essentially the same when the assay medium contained pyridoxamine as when it contained pyridoxine. In contrast to these results, the apparent

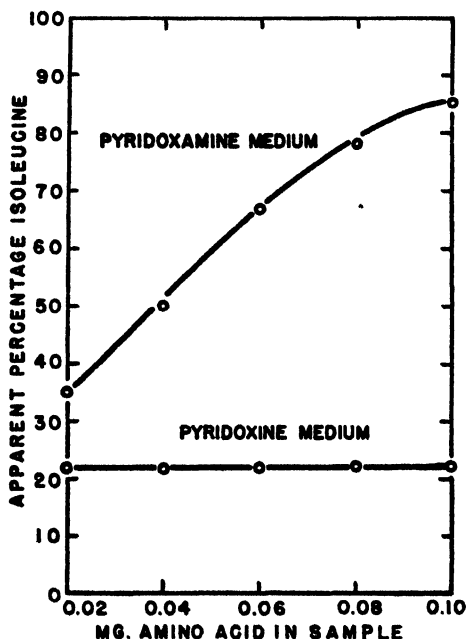


FIG. 3. Apparent isoleucine content of alloseucine-isoleucine mixtures as determined with *L. arabinosus*.

isoleucine content of the mixture of amino acid isomers used in Tests C and D (as shown in Test O of Fig. 4) was quite different for the two media. These findings indicate that the cells contained only isoleucine. Calculation of the amount of DL-isoleucine added in Test D showed that only 1.25 mg. were supplied in the medium, whereas the crop of cells contained over 5.0 mg. of L-isoleucine. The conclusions drawn from these results are that alloseucine must have been utilized and that it was converted to isoleucine before being incorporated into the cell constituents.

Vitamin B₆ is known to be specifically involved in the synthesis and metabolism of amino acids and proteins. The relationship of this vita-

min to the processes of transamination and decarboxylation is well established. Since the conversion of alloisoleucine to isoleucine would require a change in the configuration of an asymmetric carbon atom to which no amino or carboxylic acid group is attached, a postulated function of vitamin B₆ in such a reaction would appear on first consideration to involve a new function for this vitamin. However, an examination of the structural formulae of alloisoleucine and isoleucine indicates that this transfer might be accomplished by a process related to transamination or oxidative deamination. By transamination or oxidative deamination the keto analogues of alloisoleucine or isoleucine might be formed. Enolization of

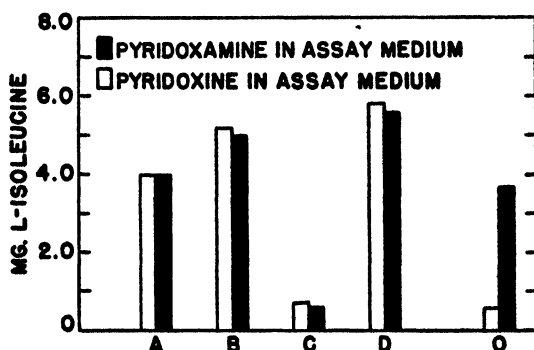


FIG. 4. Isoleucine content of *L. arabinosus* cells grown in various media. Volume of cultures, 1 liter. Incubation period, 3 days. Additions to culture media as follows: Test A, 10 mg. of DL-isoleucine and 200 γ of pyridoxine; Test B, 10 mg. of DL-isoleucine and 200 γ of pyridoxamine; Test C, 10 mg. of a mixture of alloisoleucine (87.5 per cent) and isoleucine (12.5 per cent) and 200 γ of pyridoxine; Test D, 10 mg. of a mixture of alloisoleucine (87.5 per cent) and isoleucine (12.5 per cent) and 200 γ of pyridoxamine; Test O, assay of the crystalline amino acid mixture used in Tests C and D.

either compound would, then, give an identical structure. The keto analogue might be reconverted to isoleucine through transamination.

As a result of the work of Snell *et al.* (8), it is recognized that pyridoxamine is the active form of vitamin B₆ for *L. arabinosus*. The activity of pyridoxine for this organism is due to conversion of minute amounts of pyridoxine to pyridoxamine by reaction with constituents of the medium during autoclaving or by conversion during the growth of the organism. An interpretation of the difference of behavior between pyridoxine and pyridoxamine in the present investigation is that when pyridoxine is used in the media the minute quantities of pyridoxamine formed are not sufficient to provide for the utilization of alloisoleucine, but are sufficient for certain other requirements of this organism.

SUMMARY

1. *Lactobacillus arabinosus* 17-5 can utilize alloisoleucine in mixtures of alloisoleucine and isoleucine when the medium contains pyridoxamine, but not in the absence of this vitamin. The extent of utilization is not constant at all growth levels. With increased growth levels, resulting from adding larger amounts of the mixed isomers to the tubes, the utilization of the allo isomer increases progressively.

2. Under the conditions studied, neither *Leuconostoc mesenteroides* P-60 nor *Streptococcus faecalis* R could utilize alloisoleucine.

3. The evidence indicates that the alloisoleucine utilized by *L. arabinosus* was converted to isoleucine before being incorporated into the cellular constituents.

4. A postulated mechanism for the conversion of alloisoleucine to isoleucine and the possible function of vitamin B₆ in this conversion is presented.

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ETHANOLIC FRACTIONATION OF BOVINE TESTICULAR HYALURONIDASE

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Studies on the fractionation of blood serum by Cohn *et al.* (1) have demonstrated that under precisely controlled conditions reproducible fractionation of a complex mixture of proteins can be accomplished by the use of ethanol. This paper reports the application of this method to the separation of hyaluronidase from testicular extract. A variety of experimental conditions involving variation of pH, ionic strength, and protein concentration was employed in order to ascertain optimum conditions for purification. Enzyme purity was followed in terms of activity per mg. of nitrogen.

EXPERIMENTAL

Crude hyaluronidase was extracted from bovine testes,¹ and preliminary concentration by fractional precipitation from ammonium sulfate was carried out as previously described (2). The crude concentrates contained about 2000 units per mg. of protein nitrogen. The enzyme was assayed in terms of arbitrary hyaluronidase units defined by the turbidity-reducing method of Dorfman and Ott (3). Protein nitrogen was determined by micro-Kjeldahl digestion and nesslerization. Samples contaminated with ammonium salts were dialyzed before digestion.

Low temperatures were rigorously maintained whenever the enzyme was in contact with ethanol to prevent inactivation. At 0° the enzyme could be recovered in 80 to 100 per cent yields from solutions containing up to 0.3 mole fraction of ethanol. Precooled ethanol was added at a fairly rapid rate to cold protein solutions with vigorous stirring (motor). The suspensions of precipitated protein were allowed to stand in the cold for 1 hour before filtration or centrifugation. Longer periods of standing altered the yields significantly.

The protein solutions were prepared in dilute buffers at concentrations of 0.5, 1.0, and 2.0 mg. of total protein per ml. Increasing protein concen-

¹ Bovine testes were supplied by Armour and Company, Chicago, Illinois, through the courtesy of Dr. Edwin E. Hays.

trations as high as 5 and 10 mg. per ml. slightly increased the amount of enzyme and total protein precipitated at lower ethanol concentrations, as shown in Table I, but were less favorable for the purification of the enzyme. Fractionations were therefore usually carried out at lower protein concentrations in spite of the obvious disadvantage of handling much larger volumes.

pH control was maintained by suspending all samples in 0.022 M phosphate-citrate buffers. Maximum precipitation of the enzyme occurred at lower ethanol concentrations in the vicinity of pH 5.2. This observation (Table II) was consistent with the reported isoelectric point of pH 5.4 (4). At pH 4.5 and below and at pH 7.0 and above, total enzyme recoveries decreased rapidly. Thus the useful pH range appeared to be limited from pH 4.5 to 6.5.

TABLE I

Differential Precipitation of Hyaluronidase at Varying Protein Concentrations pH 5.2; ionic strength 0.10.

Concentration of ethanol	1 mg. protein per ml.			2 mg. protein per ml.			5 mg. protein per ml.		
	Enzyme yield	Protein yield	Ratio, Enzyme Protein	Enzyme yield	Protein yield	Ratio, Enzyme Protein	Enzyme yield	Protein yield	Ratio, Enzyme Protein
<i>mole fraction</i>	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	
0.10	17	18	0.9	27	25	1.1	33	33	1.0
0.15	76	31	2.5	53	32	1.7	41	27	1.5
0.20	8	6	1.3	5	3	1.7	3	3	1.0
0.30		6		5	4	1.2	1	4	0.3

Ionic strengths were calculated for the buffered solutions before addition of ethanol and were varied from 0.05 to 0.30 by the addition of sodium chloride, since the chloride ion seemed to have a stabilizing effect on purified enzyme solutions. Typical data, illustrated in Table III, showed that increasing ionic strengths generally increased the solubilities of both enzyme and other proteins, resulting in precipitation only at higher ethanol concentrations. Above 1 per cent NaCl ($\mu = 0.20$) significant losses of enzyme activity become increasingly apparent.

These preliminary experiments demonstrated that under the explored and specified conditions solubility of both the enzyme and the inactive components increases with decreased ethanol concentration, with decreased protein concentration, with increased ionic strength, and with increased divergence from the isoelectric point. Study of the fractionation data, as illustrated in Table II, suggested that the enzyme could be precipitated

first at pH 6.5. Selected fractions from this step could then be precipitated at pH 4.5, inactive protein being removed at the lower ethanol concentrations, leaving the enriched enzyme fractions to precipitate at the higher concentrations. Accordingly, the following procedure was selected as the most efficient means for increasing the enzyme purity from about 2000 to 100,000 units per mg. of N in only two or three fractionation steps.

Fractions of the enzyme (approximately 2000 units per mg. of N) partially purified by ammonium sulfate precipitation were suspended in 0.022 M phosphate-citrate buffer at pH 6.5. Sodium chloride was added

TABLE II

Differential Precipitation of Hyaluronidase from Ethanol-Water Mixtures at Various pH Values*

Total protein concentration 0.5 mg. per ml.; ionic strength 0.10.

pH 4.0				pH 4.5			pH 5.2			pH 6.0			pH 6.5		
Concentration of ethanol	Enzyme yield			Enzyme yield			Enzyme yield			Enzyme yield			Enzyme yield		
	Enzyme yield	Protein yield	Enzyme Protein	Enzyme yield	Protein yield	Enzyme Protein	Enzyme yield	Protein yield	Enzyme Protein	Enzyme yield	Protein yield	Enzyme Protein	Enzyme yield	Protein yield	Enzyme Protein
mole fraction	per cent	per cent		per cent	per cent		per cent	per cent		per cent	per cent		per cent	per cent	
0.1	3	40	0.1	7	32	0.2	0	0		0	0		0	0	0
0.15	3	11	0.3	7	16	0.4	2	3	0.6	0	0		0	0	
0.20	4	12	0.3	6	9	0.7	52	51	1.0	6	3	2.0	0	0	
0.25	5	7	0.7	24	11	2.2	41	31	1.3	85	39	2.2	69	24	3.0
0.30	24	14	1.7	25	13	2.0	8	11	0.3	8	39	0.2	22	55	0.4

* This sample had been previously precipitated from 0.25 mole fractionation of ethanol; pH 5.2; ionic strength 0.20; enzyme purity 5000 units per mg. of N.

to give a total ionic strength of 0.10 (0.28 per cent). The suspension was adjusted to 1 mg. of protein N per ml. and dialyzed against the phosphate-citrate buffer containing sodium chloride for several days at 0°. Ethanol, precooled in a freezing bath, was slowly added to a concentration of 0.1 mole fraction. After standing for 1 hour, the precipitate was removed by refrigerated centrifugation or by filtration through paper pulp at 0°. The precipitate was immediately dissolved in buffer solution and assayed for hyaluronidase and protein nitrogen. Ethanol was again added to the filtrate to reach a concentration of 0.15 mole fraction, the precipitate was removed, and addition of ethanol to the filtrates was continued to secure precipitates at 0.2, 0.25, and 0.30 mole fractions of ethanol. Filtrates from 0.30 mole fraction usually contained negligible amounts of enzyme.

The average results of thirteen batches, all in fairly good agreement, are shown in Table IV. About 70 per cent of the enzyme was usually recovered at 0.20 mole fraction or less, while only 18 per cent of the total protein appeared in these precipitates. About 80 per cent of the inactive proteins were thus eliminated under these conditions.

It should be emphasized that considerable variation from the mean values in Tables IV and V may be expected with different batches of crude enzyme preparation. For instance, consistently lower enzyme recoveries in the 0.1 mole fraction and correspondingly higher recoveries in the 0.15 and 0.20 mole fraction precipitates were found in five runs on a crude extract of frozen testes that had been in storage for many months. In

TABLE III

Differential Precipitation of Hyaluronidase from Ethanol-Water Mixtures at Different Ionic Strengths*

Total protein concentration 0.5 mg. per ml.; pH 5.2.

Concentration of ethanol	Ionic strength 0.10			Ionic strength 0.20			Ionic strength 0.30		
	Enzyme yield	Protein yield	Ratio, Enzyme Protein	Enzyme yield	Protein yield	Ratio, Enzyme Protein	Enzyme yield	Protein yield	Ratio, Enzyme Protein
	per cent	per cent		per cent	per cent		per cent	per cent	
0.1	22	20	1.1 *	5	14	0.4	3	12	0.3
0.15	34	12	2.9	9	12	0.8	5	16	0.3
0.20	20	26	0.8	33	30	1.1	21	30	0.7
0.25	3	9	0.3	19	11	1.7	20	15	1.3
0.30	1	3	0.3	14	4	3.5	13	4	3.2

* This sample was a crude preparation, was precipitated once with ammonium sulfate, and had no previous alcohol fractionation; the enzyme purity was 750 units per mg. of nitrogen.

general, variation can be anticipated in enzyme recovery because of its unstable nature and consequent hazard of denaturation in handling. The total protein values, on the other hand, have been quite consistent since they have been measured as Kjeldahl nitrogen, which does not reflect the losses due to denaturation.

Inspection of Table IV obviously indicated the selection of the precipitates from 0.10, 0.15, and 0.20 mole fractions for the second ethanol fractionation. These fractions contained 7000 to 10,000 units per mg. of N.

When large quantities of material were under treatment, the low grade precipitates from 0.25 mole fraction (several batches) were reserved until sufficient quantities had been accumulated for profitable refractionation.

These selected precipitates from the first ethanol fractionation were

dissolved in 0.022 M phosphate-citrate buffer and titrated to pH 4.5 with HCl. Total protein concentration was adjusted to 1 mg. of protein per ml. The average enzyme purity was near 9000 units per mg. of protein N. The ionic strength was adjusted to 0.10 by the addition of NaCl to a con-

TABLE IV

First Fractional Precipitation of Hyaluronidase and Total Proteins from Ethanol-Water Mixtures at pH 6.5*

Ionic strength 0.10; total protein concentration 1 mg. per ml. Average of thirteen runs.

Concentration of ethanol	Enzyme yield	Protein yield	Ratio, Enzyme Protein
<i>mole fraction</i>	<i>per cent</i>	<i>per cent</i>	
0.10	19.7	5.9	3.3
0.15	27.2	5.3	5.1
0.20	23.4	6.8	3.4
0.25	11.5	16.5	0.7

* These samples were selected from the best ammonium sulfate fractions and had no previous ethanol treatment. The enzyme purity was 2000 units per mg. of N.

TABLE V

Second Fractional Precipitation of Hyaluronidase and Total Proteins from Ethanol-Water Mixtures*

pH 4.5; ionic strength 0.10; and total protein concentration 1 mg. per ml. Average of fifteen runs.

Concentration of ethanol	Enzyme yield	Protein yield	Ratio, Enzyme Protein
<i>mole fraction</i>	<i>per cent</i>	<i>per cent</i>	
0.10	22.0	51.7	0.4
0.15	12.3	5.6	2.1
0.20	11.7	2.9	4.2
0.25	21.8	2.9	8.0
0.30	9.0	2.1	4.9

* These samples were 0.1, 0.15, and 0.20 mole fraction precipitates from the first ethanol fractionation, Table IV. The enzyme purity was 6000 to 13,000 units per mg. of N. The average was 10,000 units per mg. of N.

centration of 0.28 per cent. Cold ethanol was added as described and the precipitates were removed and assayed, with the results shown in Table V.

In this step 56 per cent of the protein and about 35 per cent of the enzyme precipitated at the lower ethanol concentrations of 0.10 and 0.15

mole fractions. As a rule, these fractions were discarded. However, appropriate refractionation procedures can be employed to recover a part of the enzyme. Over 40 per cent of the enzyme usually appeared in 0.20, 0.25, and 0.30 mole fraction precipitates, accompanied by only 8 per cent of the total protein. The different fractions varied from 50,000 to 100,000 units per mg. of N, depending upon the purity of the starting material. Modest yields of the purest enzyme were most easily obtained by selecting only the best fraction from the first ethanol treatment, 12,000 to 13,000 units per mg. of N, for the second ethanol fractionation. Thus 6 per cent of the original crude enzyme could be brought to 100,000 units per mg. of N in only two fractionation procedures.

The greater part of the lower grade fractions from the first and second ethanol treatments could be brought to a purity of 100,000 units per mg. of N by a third ethanol treatment, best carried out at pH 5.2. Data for these steps followed much the same pattern as that illustrated in Tables IV and V.

Preliminary electrophoretic studies on enzyme of 100,000 units per mg. of N indicated heterogeneity. One fraction was isolated by electrophoresis which showed an activity of approximately 200,000 units per mg. of N.

Enzyme containing 100,000 units per mg. of N was also fractionated by ammonium sulfate. Fractions with activities near 200,000 units per mg. of N were obtained but inconsistent data from three trials indicated that these samples were unstable and rapidly lost activity. Available evidence suggested that optimum conditions for stability of the highly purified enzyme should be ascertained before reliable data can be collected.

SUMMARY

A procedure has been detailed for a 50-fold purification of the enzyme hyaluronidase by fractional precipitation from ethanol-water mixtures at controlled ionic strength, protein concentration, temperature, and pH. Two or three fractionation steps were sufficient to increase the purity of the enzyme from 2000 units per mg. of N in the crude preparations to 100,000 units per mg. of N in the purified product, with yields of 15 per cent of the crude enzyme.

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STUDIES ON THE DIVERSITY AND THE NATIVE STATE OF DESOXYPENTOSE NUCLEIC ACIDS*

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The investigator of naturally occurring high polymers is faced with the necessity of deciding whether preparations of an ostensibly similar composition and physical appearance, but isolated from different cellular sources, are identical, and whether they are representative of the state in which these substances occur in the living cell. Strictly speaking, no compound, once it is isolated from the cell, can be considered as native. However, the series of degradative changes to which it may be exposed in the course of its isolation will usually be gradual; and, while it may not yet be possible to define the perfect compound, the badly degraded one will, as a rule, be recognized. In compounds, such as the nucleic acids, that occur in the cell in combination with proteins, most likely as the prosthetic groups of conjugated proteins, the danger of secondary changes attending their liberation is particularly great, since whatever forces had anchored them to the protein must now have become free to interact.

It has been pointed out in recent reports from this laboratory (1, 2) that the recognition of differences between desoxypentose nucleic acids derived from different sources is intrinsically much more difficult than it is in the case of proteins. Nevertheless, significant differences in purine and pyrimidine composition have been discovered in the desoxypentose nucleic acids from phylogenetically remote species, such as ox (1), yeast, and tubercle bacilli (3), and even from less distant species, *e.g.* ox and man (4). It may be assumed that the number of distinct desoxypentose nucleic acids is much larger than could be revealed at the present time by constitutional analysis. For this reason, a search for supplementary criteria of differentiation and characterization has been initiated, the first results of which are submitted here.

In many of their outward characteristics, the highly polymerized desoxypentose nucleic acids from different cells resemble each other very much. The elementary composition, the degree of viscosity, and the electrophoretic and diffusion behavior could hardly serve as means of distinction

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and only slight differences in the centers of absorption in the ultraviolet region have been observed (1, 3, 5). For this reason, the comparison of controllable changes produced in the intact nucleic acids offers more promise as a supplement to the differentiation between nucleic acids by constituent analysis than does the comparison of their physical properties.

One of the most outstanding properties of cautiously prepared desoxypentose nucleic acids (1, 5, 6-8) is the high viscosity of their solutions. The addition of acid or alkali to calf thymus nucleic acid produces a very considerable drop in viscosity (6, 9-13). This is most likely due to a far-reaching depolymerization of the molecule, rather than to the diminished interaction between the individual fibers, as has also been proposed (14). On readjustment to pH 7 the viscosity increases again (11, 12), but it can be shown, in agreement with previous findings (15, 16), that this is not a true reversal to the original state. The critical limits between which the high viscosity of solutions of the sodium salt of desoxypentose nucleic acid of calf thymus remained constant were reported as pH 5.6 and 10.9 (12).

It appeared of interest to extend the observations on the dependence upon pH of the viscosity of desoxypentose nucleic acid solutions to comparable preparations from a source very remote from calf thymus, namely yeast (5). In addition to the fact that this desoxypentose nucleic acid differs in its composition from that of calf thymus (3), recent findings on the properties of the desoxypentose nuclease of yeast (17) made the study of the highly polymerized yeast desoxypentose nucleic acid particularly attractive. The depolymerizing enzyme from yeast exerted its optimal activity below pH 6; at pH 5.6 it still retained as much as 98 per cent of its maximal potency. The pancreatic desoxyribonuclease, on the other hand, had its optimum at pH 7.4, about 90 per cent of its activity being lost at pH 5.6.

Thus, the pH optimum of the enzyme from pancreas lies within the normal pH range of the mammalian cell, at a point at which the ox desoxypentose nucleic acid is very stable with respect to viscosity changes. On the other hand, at the pH optimum of the yeast desoxypentose nuclease (pH 5.8 to 6),¹ the yeast desoxypentose nucleic acid in the living cell would be dangerously near the threshold of viscosity drop (pH 5.6), unless the nucleic acids of yeast and calf thymus behaved entirely differently in their

¹ There is, of course, no reason to assume that the pH optimum of an intracellular enzyme always reflects the average pH of the cell from which it derives. Preparations from ox tissues with an optimal desoxyribonuclease activity at pH 4.5 have been described recently (18). It should, however, be noted that the exposure to pH 4.5 induces by itself a drop in viscosity of about 20 per cent in very highly polymerized preparations of calf thymus desoxyribonucleic acid.

stability to depolymerization by acid. The experiments described here show the latter actually to be the case: the desoxypentose nucleic acid of yeast is stable down to pH 3.7 (Fig. 1).

To what extent these stability differences reflect the differences in purine and pyrimidine composition found in these two nucleic acids cannot be said. If, as Gulland *et al.* (15) have suggested, the macromolecular character and the stability of undegraded desoxypentose nucleic acids are maintained by hydrogen bonds between the amino groups and the enolic hydroxyls of the purines or pyrimidines, divergences in the proportions, and even more in the sequence, of these components would most probably

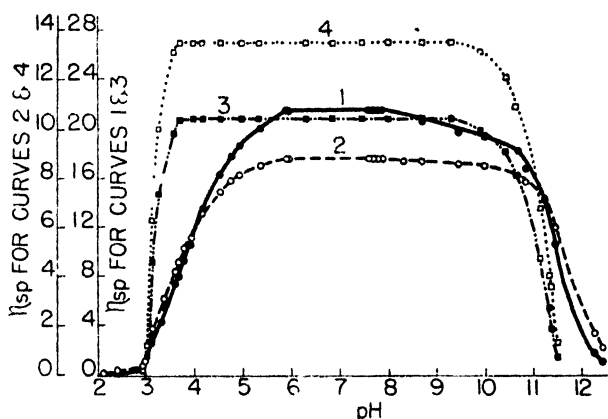


FIG. 1. Stability of desoxypentose nucleic acids of calf thymus (Preparation T-1) and of yeast (Preparation Y-1) to acid and alkali. The solutions in 0.05 *M* aqueous NaCl had the following concentrations: Preparation T-1, 0.25 per cent (Curves 1 and 2); Preparation Y-1, 0.39 per cent (Curves 3 and 4). The specific viscosities are plotted as the ordinate; the abscissa indicates the pH. Curves 1 and 3, Ostwald-Fenske viscosimeter under gravity; Curves 2 and 4, under constant applied pressure.

influence the stability of the respective nucleic acids. It is, perhaps, significant that the desoxypentose nucleic acids of ox and yeast differed much less in their behavior towards alkali than towards acid.

Studies of the average pH of yeast, which is known to be able to multiply in relatively very acidic media, have shown that this cell tolerates a rather wide range of cytoplasmic pH changes from pH 6.6 down to about pH 4.5 (19-23). If this applies also to the cell nucleus, the higher degree of acid stability of the yeast desoxypentose nucleic acid could be regarded as fulfilling a necessary function, especially if the integrity of the desoxypentose nucleic acid is a condition for the unchanged transmission of hereditary problems.

This difference in stability toward depolymerization by acid is not the

only distinction observed. The depolymerization of calf thymus desoxyribonucleic acid, as followed by viscosity changes, takes place over a wide range, from pH 5.6 to 2.5, whereas in the yeast compound the range is narrow, pH 3.7 to pH 3, as shown in Fig. 2. The shapes of the depolymerization curves of the two nucleic acids at elevated pH values are rather similar (Fig. 1), but, whereas in the product from calf thymus considerably more equivalents of alkali than of acid are necessary for the production of the same decrease in viscosity, the required equivalents of alkali and of acid are nearly the same in the case of the yeast desoxyribonucleic acid (Fig. 3).

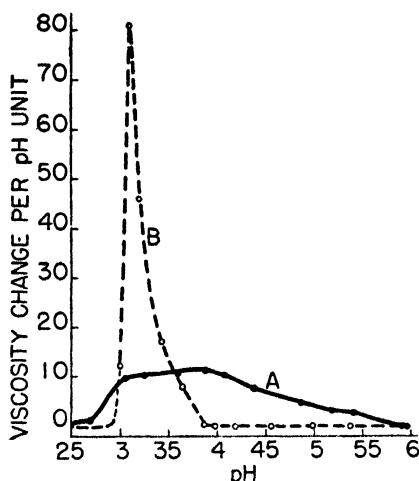


FIG. 2. The viscosity changes under gravity per pH unit (viscosity gradients) are plotted as the ordinate, the pH values as the abscissa. Curve A, Preparation T-1 from calf thymus; Curve B, Preparation Y-1 from yeast.

The second part of this paper deals with the problem of the native state of a desoxypentose nucleic acid. The criteria of integrity of a macromolecular substance of natural origin are not easy to define, as has been pointed out before, but as regards the desoxypentose nucleic acids, certain features can be described. If, in the course of its isolation, the nucleic acid is protected from the action of high temperatures, acid, and alkali (6), as well as that of depolymerizing enzymes (5, 7, 8), a preparation is obtained possessing certain unique properties. It has a *very high and, within the specimen, uniform molecular weight*, as shown by diffusion experiments (11, 24, 25), sedimentation in the ultracentrifuge (24-28), and determinations of viscosity and streaming birefringence (29). The character of monodispersity is lost if the specimen is prepared under very mild

conditions (6) but without the avoidance of partial enzymatic attack in the course of isolation. Both the extent and the uniformity of the molecular weight are affected if the isolation is carried out under degradative conditions (26, 30), or if the preparation is exposed subsequently to degradation by chemical or physical means (11, 24, 27, 31, 32). Even if in such damaged preparations repolymerization to a high average molecular weight takes place, the monodispersity of the preparation is not reestablished (11, 24).

Another characteristic property of "undenatured" desoxypentose nucleic acids is their *anomalous amphoteric behavior* on titration (15, 33). A third distinguishing feature may be seen in the *high and stable asymmetry* of the molecules. Solutions of undegraded desoxypentose nucleic acid exhibit

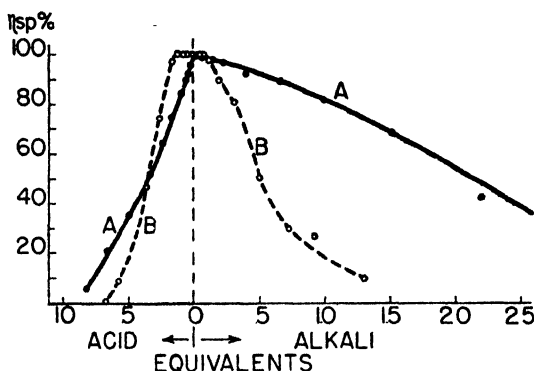


FIG. 3. The specific viscosities, corresponding to Curves 2 and 4 in Fig. 1, as per cent of the initial viscosity, are plotted as the ordinate. The equivalents (per mole of P) of acid or alkali necessary for a given change in viscosity are plotted as the abscissa. Curve A, thymus desoxyribose nucleic acid, Preparation T-1; Curve B, yeast desoxyribose nucleic acid, Preparation Y-1.

double refraction of flow (12, 29, 34, 35) and very considerable viscosity. The conditions under which the viscosity is destroyed and ostensibly restored have already been mentioned in the beginning of this paper.

In this connection, some experiments presented here, that indicate an important difference between solutions of an undegraded and presumably native desoxypentose nucleic acid and those of preparations degraded under conditions such as to preserve their ability of repolymerization, may be of interest. The viscosity of a solution of the intact preparation has been found to be extremely constant; it exhibited no thixotropy. This characteristic was lost irreversibly when the preparation, degraded by acid, alkali, or heat, was permitted to repolymerize (Figs. 4 and 5); the viscosity thus reestablished was highly thixotropic. (For a brief

discussion of the interesting phenomenon of thixotropy, see Freundlich (36).) An essentially similar distinction may be seen in the finding that the viscosity of the products that had undergone repolymerization was affected by thermal vibrations at temperatures at which the original preparation was entirely stable. This type of gel-sol transformation could, in analogy to the term thixotropy, be designated as "thermotropy" (Fig. 6). The phenomena described here were observed with desoxypentose nucleic acid preparations from calf thymus and from yeast, depolymerized not only by acid or alkali but also by heat; they do not

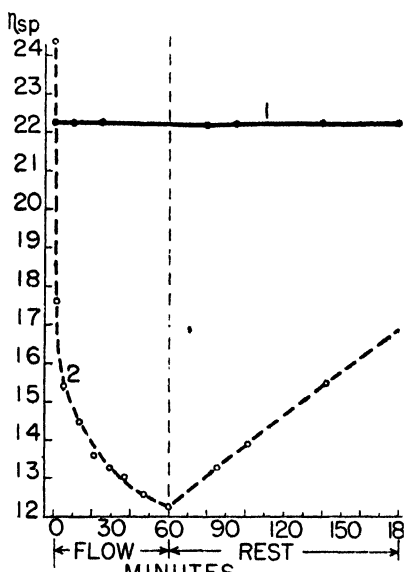


FIG. 4. Viscosity behavior of undegraded and degraded calf thymus desoxypentose nucleic acid (Preparation T-1). The specific viscosities are plotted as the ordinate. The abscissa indicates the net duration of forced flow or of rest. Curve 1, original preparation; Curve 2, acid-degraded preparation that had repolymerized.

apply, however, to the products obtained by the action of desoxyribonuclease.

Future work will probably reveal other characteristic properties of intact desoxypentose nucleic acids, one of the most important already adumbrated being their biological activity (37). In the meantime, the criteria discussed here may, in addition to the chemical studies presented in other publications from this laboratory, assist in the differentiation between desoxypentose nucleic acids of different origin and of different degrees of preservation. These findings can be considered as additional evidence that the original preparations of desoxypentose nucleic acids

unique physical properties (non-thixotropic viscosity, regular temperature-viscosity relationship) which, once lost, cannot be regained by re-polymerization. They also are, perhaps, of more general interest, inasmuch as they extend the concept of irreversible denaturation from the field of proteins to that of another group of macromolecules, the nucleic acids.

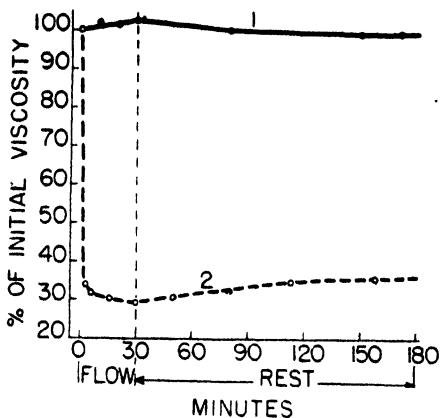


FIG. 5

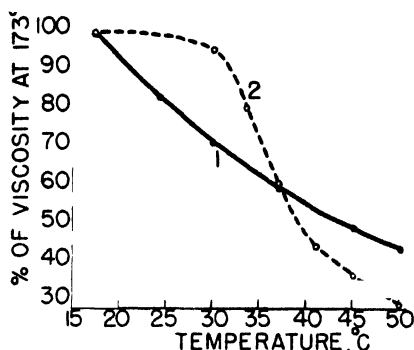


FIG. 6

FIG. 5. Viscosity behavior of undegraded and degraded yeast desoxypentose nucleic acid (Preparation Y-1). The specific viscosities as per cent of initial viscosity are plotted as the ordinate. See Fig. 4 for other explanations.

FIG. 6. Viscosity behavior of undegraded and heat-degraded calf thymus desoxypentose nucleic acid (Preparation T-1) as a function of the temperatures of measurement. The specific viscosities as per cent of viscosity at 17.3° are plotted as the ordinate. The abscissa indicates the temperatures at which the determinations were performed. Curve 1, original preparation; Curve 2, heat-degraded preparation that had repolymerized.

EXPERIMENTAL

Stability of Desoxypentose Nucleic Acids of Calf Thymus and Yeast to Acid and Alkali

Material—Two desoxypentose nucleic acid preparations from each yeast sample and calf thymus were examined. The *desoxyribonucleic acid of yeast* listed as Preparation Y-1 was isolated (5) as the highly polymerized sodium salt and analyzed (3) by the procedures described previously. Viscosity measurements on several preparations of this type have been reported before (5). The preparation employed contained less than 0.3 per cent of protein (38). A 0.39 per cent solution of Preparation Y-1 in 0.05 M aqueous sodium chloride was used in the experiments; it had a viscosity of $\eta_{sp} = 20.8$ (under gravity). Another preparation from yeast,

designated as Preparation Y-2, was obtained by an essentially similar procedure, but it represented a fraction precipitated by alcohol as short threads and consisted of partly depolymerized material. When used as a 0.7 per cent solution in 0.05 M aqueous NaCl, it exhibited a rather low viscosity, $\eta_{sp} = 1.8$ (under gravity).

The preparation and composition of the sodium salt of the *desoxyribonucleic acid of calf thymus* listed here as Preparation T-1 has been discussed in a previous publication (1) as Preparation 3. It was obtained by essentially the same methods as were the preparations from yeast, contained less than 0.5 per cent of protein, and was used as a 0.25 per cent solution in 0.05 M NaCl with an $\eta_{sp} = 21.3$ (under gravity). Another similarly prepared specimen from calf thymus (Preparation T-2) was employed as a 0.17 per cent solution in 0.05 M NaCl with an $\eta_{sp} = 61$ (under gravity).

Methods—The determinations of *viscosity* were carried out in either of two ways. (a) An Ostwald-Fenske viscosimeter, having a capillary 7.2 cm. long and 0.8 mm. in diameter, was used. The time necessary for 1.75 cc. of water to pass through this capillary by gravity was 12.2 seconds at 24.8°. (b) Measurements were also performed with the same viscosimeter, but under constant pressure (applied pressure of 10 cm. of water). The velocity gradients β (39, 40) thus obtained varied between 3840 for water and 402 for the most viscous nucleic acid solutions tested. It has been shown (34) that with such values of velocity gradient and at the concentrations employed the relative viscosity figures reach nearly constant values independent of β . The total volume of liquid in the viscosimeter was 4 cc., the temperature 24.8°.

The measurements of *hydrogen ion concentration* were carried out in the Beckman pH meter with a glass micro electrode (No. 290M) up to pH 11.5 and with a larger glass electrode above this value. Since the salt concentration must be kept constant in viscosity measurements, the samples used for pH determinations cannot be returned to the main solution because of possible contamination with KCl from the calomel electrode. The micro electrode offers the advantage of requiring no more than negligible amounts of liquid. In the region above pH 11.5, where a glass electrode of standard size had to be employed, the viscosity of the nucleic acid solution had fallen to a value so low that the introduction of traces of additional electrolyte produced no noticeable error in the viscosity measurements.

A micrometric burette was used in the *titrations*. Accurately standardized solutions of hydrochloric acid and sodium hydroxide were used. Their approximate normalities were 0.1 and 0.5 for HCl, and 0.1, 0.5, 1.0, and 2.0 for NaOH. In order to avoid undue dilution of the solutions of

desoxypentose nucleic acid in the course of titration, increasingly stronger acid or alkali was used. Although the increase in final volume did not exceed 2 per cent of the initial volume, correction was made for the error in the viscosity measurements due to this dilution. This was done by means of model experiments in which records were made of the viscosity changes in a control portion of the nucleic acid solution to which corresponding quantities of an NaCl solution of the same ionic strength had been added. The acid or alkali was introduced very slowly, with vigorous stirring, and in small portions not exceeding 0.015 cc., in order to avoid local overconcentrations.

Experimental Arrangement—The nucleic acid solutions described in the beginning of this section were divided into two parts, one to be used for the viscosity titration in the acid range, the other in the alkaline range. A 5 cc. portion of the solution was placed in the titration vessel, a minute amount (less than 0.01 cc.) was withdrawn for the measurement of pH, and a 4 cc. sample for the determination of viscosity. The latter was subsequently returned to the titration vessel, the pH rechecked, and the quantity of acid or alkali required to reach the next point was added from the micro burette with the precautions described above. Viscosity and pH determinations were then repeated. As a rule, at each stage one viscosity measurement was carried out within 1 minute after the addition of acid or alkali, another 30 minutes later. No appreciable differences between these two measurements were, in fact, ever found.

Results—The effects of acidity on the viscosity behavior of two of the desoxypentose nucleic acids compared here (Preparations T-1 and Y-1) are shown in Fig. 1. Within the range studied, these effects appear to be instantaneous, in accordance with previous findings (13). The differences in stability of the calf thymus and yeast preparations are clearly indicated: a gradual viscosity decline of the first, beginning at pH 5.6 to 5.8, and a steep viscosity drop of the latter at pH 3.7. This difference in the respective rates of depolymerization is brought out in Fig. 2, which shows the viscosity gradients for both nucleic acids; *i.e.*, the rates of viscosity changes per pH unit. The curve for the desoxypentose nucleic acid of calf thymus is low and flat, extending over a considerable range of pH (pH 5.8 to below pH 2.5); the gradient for the compound from yeast is 0 above pH 3.7 and below pH 2.9, but at pH 3.1 it sharply reaches a very high value that is about 7 times that of the highest figure for the product from calf thymus.

The respective stabilities to alkali are also illustrated in Fig. 1. No considerable differences are exhibited by the two compounds in this region, though the nucleic acid from yeast seems to have a narrower range of viscosity decline. This figure can be interpreted as showing that calf

thymus desoxypentose nucleic acid, in contrast to that of yeast, is less stable on the acid than on the alkaline side. This is demonstrated in a different manner in Fig. 3, which represents what may be called the viscosity titration curves; i.e., the equivalents of acid or alkali necessary for the production of a given percentage decrease in viscosity. This diagram contrasts the relatively symmetric curve obtained with yeast desoxypentose nucleic acid with the highly asymmetric one given by the substance from thymus. It is, however, somewhat more difficult to interpret than are Figs. 1 and 2 which limit themselves to a feature characteristic of the intact molecule, since here not only the equivalents required for depolymerization are involved, but also those necessary for the titration to a given pH of the acidic and basic groups present in the nucleic acid (15) regardless of its state of polymerization.

When the experiments were repeated with the solutions of two other preparations from yeast and thymus respectively (Preparations Y-2 and T-2), which have been mentioned before, the viscosity (under gravity) of the partly depolymerized Preparation Y-2 remained unchanged down to pH 3.8 and then dropped abruptly, with a particularly high gradient at pH 3.25. The viscosity (under gravity) of Preparation T-2 on the other hand, began to decrease gradually at pH 5.8; all other features also were similar to the experiment recorded in Fig. 1.

Thixotropy of Degraded Desoxypentose Nucleic Acids

Material and Methods—The experiments were performed with the desoxypentose nucleic acids of calf thymus and of yeast (Preparations T-1 and Y-1) following depolymerization by acid, alkali, or heat. In order to conserve space, only the experiments with the nucleic acids depolymerized by acid will be described in detail. Solutions of thymus nucleic acid (0.25 per cent) and of the yeast nucleic acid (0.39 per cent) in 0.05 M aqueous NaCl were employed as the starting material. They were adjusted at room temperature to pH 2.98 (calf thymus) or pH 2.4 (yeast), when their specific viscosities (determined by gravity in the Ostwald-Fenske viscosimeter specified above) dropped from 21.4 to 0.5 (calf thymus) or from 25.1 to 0.2 (yeast) (compare Fig. 1). The original solutions of the same nucleic acids that had not undergone depolymerization served as the controls.

Experimental Arrangement—The solutions were, within 30 minutes after the addition of acid, neutralized by the careful addition of N sodium hydroxide with thorough mixing. They were then (protected by 0.002 per cent ethyl mercurithiosalicylate) kept at room temperature for 3 days (calf thymus) or 1 day (yeast). A 0.35 cc. portion of the solution was introduced carefully into a micro viscosimeter of the Ostwald type

(capillary 14 cm. long, 0.45 mm. diameter; water value 21.8 seconds at 24.8°); the first viscosity determination was carried out 1 hour later. Following this measurement, the liquid was rapidly forced upward and downward in the capillary by the alternate application of air pressure (100 cm. of Hg) to one or the other stem opening (with a frequency of 10 to 15 alternations per minute). The velocity gradient during this operation was of the order of 10,000. The forced flow was continued for a net period of 1 hour (thymus) or $\frac{1}{2}$ hour (yeast), interrupted only by occasional viscosity measurements. The liquid then was allowed to remain stationary, except for the time required by viscosity determinations. The solutions serving as controls underwent the same treatment.

Results—The repolymerization and thixotropy experiments are summarized in Fig. 4 (calf thymus) and Fig. 5 (yeast). They show that the undegraded nucleic acids exhibited a remarkably constant viscosity, despite the forced movement at high speed to which they were subjected. The calf thymus preparation that had been degraded by acid repolymerized upon neutralization and storage from $\eta_{sp} = 0.5$ to $\eta_{sp} = 24.4$; the yeast preparation, similarly treated, from 0.2 to 17.4. The regained viscosities were, however, of a highly thixotropic character and dropped to approximately one-half and less than one-third, respectively, when the solutions of degraded calf thymus and yeast preparations were made to move rapidly and continuously.² Similar effects were observed with preparations from both sources that had been degraded by alkali.

Stability to Thermal Vibrations

A 0.3 per cent solution of highly polymerized calf thymus nucleic acid (Preparation T-1) in 0.1 M sodium citrate buffer of pH 7.4 ($\eta_{sp} = 30.3$ at 30° in the Ostwald-Fenske viscosimeter described before) was subjected to depolymerization by being kept at 86° for $1\frac{1}{2}$ hours. The specific viscosity (at 30°) of the solution had dropped to 3.9. When kept at 30° for 1 hour, the material repolymerized to $\eta_{sp} = 22.5$. At this stage, the solution showed the thixotropic properties reported in the preceding section. The solution was stored in the refrigerator for about 60 hours and the viscosity of the repolymerized material determined at different bath temperatures. A portion of the original unheated solution containing the intact nucleic acid served as the control.

² The thixotropic properties apparently are a function of the electrolyte concentration (36). Following the removal of salt (by dialysis or precipitation with alcohol) from the solutions of the degraded nucleic acids that had repolymerized, the aqueous solutions showed no thixotropy. When NaCl was added, the thixotropy appeared at salt concentrations higher than 0.02 M, reached a maximum at 0.2 to 0.3 M, and then declined gradually.

The results are presented in Fig. 6 which compares the viscosities of the two products as a function of the temperature. The intact nucleic acid gave a regular viscosity curve agreeing closely with the well known empirical equation $\log \eta = a + (b)/(T)$, where a and b are constants. On the other hand, the nucleic acid that had depolymerized under the influence of heat and then repolymerized exhibited an S-shaped curve, indicating that between 30–41° the thermal vibrations already were sufficient to break the loose association of molecules.³

SUMMARY

Previous evidence on differences in the chemical composition of the desoxypentose nucleic acids of calf thymus and yeast is now supplemented by the demonstration of distinctions between the highly polymerized preparations themselves. While the limit of stability to acid of calf thymus nucleic acid is at pH 5.8, as shown by a gradual viscosity drop at lower pH values, the corresponding product from yeast is entirely stable down to pH 3.7 and depolymerizes extremely rapidly below this value. Other differences in the depolymerization behavior of these two nucleic acids also are discussed, as is the problem of the integrity of these substances which, when prepared very cautiously, appear to have retained several, or possibly all, of the characteristics of the native desoxypentose nucleic acid of the cell. Once depolymerized, the original nucleic acid cannot be reconstituted. Though the solutions (after degradation by acid, alkali, or heat, but not by desoxyribonuclease) are able to regain high viscosity, an artifact is formed which has a highly thixotropic character, in contrast to the constant viscosity of the original preparations. Differences in thermal stability between the intact and the heat-degraded repolymerized preparations also are discussed.

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³ It might be mentioned that this material showed no tendency to repolymerize when kept at temperatures higher than 45°; on cooling repolymerization occurred.

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RESOLUTION OF RACEMIC PHENYLALANINE

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Phenylalanine has previously been resolved by fractional crystallization of the brucine salt of formyl-DL-phenylalanine (1) or of the cinchonine salt of benzoyl-DL-phenylalanine (2). Enzymatic methods have also been used for the resolution. According to the method of Gilbert, Price, and Greenstein (3), racemic phenylalanine is resolved by subjecting its *N*-chloroacetylated derivative to asymmetric hydrolysis by a carboxypeptidase present in beef pancreas. A preparation of the enantiomorphs of DL-phenylalanine has also been reported by making use of the difference in rates of synthesis by papain of the anilides of acetyl-DL-phenylalanyl-glycine (4).

In this paper, a simple method is described, which is based upon the asymmetric hydrolysis of the isopropyl ester of DL-phenylalanine by an enzyme preparation derived from pancreas. Complete digestion of the ester yields free L-phenylalanine and the isopropyl ester of D-phenylalanine. The latter is easily separated by virtue of its solubility in ether and alcohol. The ethyl ester of DL-phenylalanine is hydrolyzed in the same manner. However, the isopropyl ester is not hydrolyzed appreciably by water at room temperature and is therefore more suitable for the purpose. Resolution of amino acids by asymmetric hydrolysis of their esters has previously been used to obtain the isomers of leucine (5), methionine (6, 7), and tryptophan (8).

EXPERIMENTAL

Isopropyl Ester of DL-Phenylalanine—100 gm. of DL-phenylalanine are suspended in 1400 ml. of an anhydrous solution of isopropyl alcohol containing 10 per cent of anhydrous hydrochloric acid. The mixture is refluxed for 2 hours. A clear solution is obtained, which is evaporated to dryness on a steam bath *in vacuo*. To the hydrochloride of the isopropyl ester thus obtained are added 300 ml. of distilled water and 500 ml. of ether. While stirring, the mixture is cooled to -10° . A 25 per cent solution of ammonium hydroxide is added until the reaction of the mixture is faintly alkaline to phenolphthalein. The ether layer is separated and

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the water solution is extracted with two portions of 250 ml. of ether. The combined ether extracts, containing the isopropyl ester, are dried over anhydrous sodium sulfate. The sodium sulfate is removed and washed with 100 ml. of anhydrous ether. The ether extracts are distilled *in vacuo*, and the fraction distilling between 128–131° at 5 mm. contains the isopropyl ester of DL-phenylalanine; yield 110 to 116 gm. (88 to 93 per cent of theory).

$C_{15}H_{17}O_2N$. Calculated, C 69.5, H 8.3, N 6.8; found, C 69.7, H 8.3, N 6.9

Enzyme Preparation—The enzyme used in this investigation was prepared from raw pancreas desiccated and defatted at 37°.¹ The pancreas

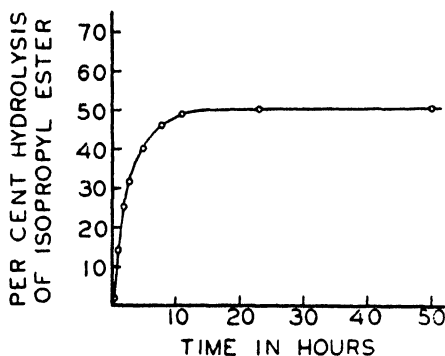


FIG. 1. The asymmetric hydrolysis of isopropyl ester of DL-phenylalanine by an enzyme preparation from pancreas. The digests consisted of 20 ml. of 0.5 per cent filtered pancreas solution and 1 gm. of the isopropyl ester of DL-phenylalanine. The reaction was performed at room temperature (23°). Hydrolysis was followed by formol titration.

powder is gently shaken in distilled water for half an hour, filtered, and the filtrate used for the resolution procedure. The rate of hydrolysis of the L ester is shown in Fig. 1. It is evident that the D ester is completely resistant for at least 50 hours.

Preparation of L-Phenylalanine—100 gm. of the isopropyl ester of DL-phenylalanine are gently shaken for 24 hours at room temperature with 400 ml. of a filtered 2.5 per cent extract of the pancreas powder. At the end of this time, there is a heavy precipitate of L-phenylalanine. While stirring, 10 ml. of 25 per cent ammonium hydroxide and 400 ml. of ether are added to the digestion mixture. The stirring is continued for an hour, when the mixture is filtered and the precipitate is washed with three 25 ml. portions of absolute alcohol.

¹ Viobin Corporation, Monticello, Illinois.

The precipitate of L-phenylalanine is further extracted by being stirred for an hour at gentle boiling temperature with a mixture of 300 ml. of ether and 100 ml. of absolute alcohol. After filtration, the precipitate is allowed to stand overnight at room temperature under 200 ml. of ether. The ether, which has evaporated, is then restored, and the mixture is stirred for an hour and filtered. After being washed twice with 50 ml. of ether, the precipitate is allowed to dry in the air.

A second crop of L-phenylalanine is obtained from the digestion mixture in the following way. The ether layer is separated and the water solution is extracted with two 100 ml. portions of ether, concentrated *in vacuo* to 150 to 200 ml., treated with one-third of its volume of 95 per cent alcohol, and chilled overnight. The precipitate is washed with two 25 ml. portions of 30 per cent alcohol and two like portions of absolute alcohol. The combined precipitates of impure L-phenylalanine should weigh 37 to 40 gm.

All of the ether extracts, containing the isopropyl ester of D-phenylalanine, are combined, dried over anhydrous sodium sulfate, and saved for the preparation of D-phenylalanine.

The impure L-phenylalanine is recrystallized from 11 times its weight of hot distilled water after treatment with norit. 0.33 volume of 95 per cent alcohol is added and the whole is chilled overnight. The crystals are washed with 30 per cent alcohol and absolute alcohol as above. The L-phenylalanine thus obtained contains 99.0 to 99.5 per cent of the L isomer and weighs 24 to 30 gm. To obtain an analytically pure sample, the L-phenylalanine is again recrystallized and washed as outlined above. The product thus obtained should weigh 19 to 23 gm. (49 to 58 per cent of theory). Analysis of a typical preparation yielded the following result.

$C_9H_{11}O_2N$. Calculated, N 8.48; found, N 8.44

$[\alpha]_D^{25} = -35.0$ to -35.2 ($c = 2$, in distilled water)

The values reported in the literature are -35.1 (1), -34.8 (3), -35.3 (9).

Preparation of D-Phenylalanine—The combined ether extracts from the preparation of L-phenylalanine are filtered, the sodium sulfate is washed with 100 ml. of anhydrous ether, and the ether is evaporated. The resulting oil is shaken for 24 hours with 100 ml. of a 5 per cent aqueous extract of the pancreas powder in order to remove any contamination with the L ester. After the addition of 5 ml. of a 25 per cent solution of ammonium hydroxide, the mixture is extracted with three 75 ml. portions of ether. The combined ether extracts are dried over anhydrous sodium sulfate and concentrated on a steam bath with the aid of a water pump. The remaining oil (weighing 38 to 42 gm.) is refluxed for 12 hours with

7 volumes of 20 per cent hydrochloric acid. The solution is evaporated to dryness *in vacuo* and for a second time after adding 300 ml. of distilled water. The hydrochloride of D-phenylalanine thus obtained is dissolved in 250 ml. of water, decolorized with 0.5 gm. of norit, and the clear solution neutralized with 25 per cent ammonium hydroxide and treated with 90 ml. of 95 per cent alcohol. The crystals which separate overnight are washed with alcohol as above and should weigh 20 to 23 gm.

The impure D-phenylalanine is recrystallized by the same technique as the L enantiomorph. The analytically pure D-phenylalanine thus obtained weighs 12 to 15 gm. (30 to 38 per cent of theory). Analysis of a typical preparation yielded the following results.

$$\text{C}_9\text{H}_{11}\text{O}_2\text{N}. \text{ Calculated, N 8.48; found, N 8.55}$$
$$[\alpha]_D^{25} = +35.0 \text{ to } +35.2 \text{ (} c = 2, \text{ in distilled water)}$$

The values reported in the literature are +35.08 (2), +34.8 (3), +34.56 (10).

SUMMARY

A simple and effective method is described for the preparation of both isomers of phenylalanine by the asymmetric hydrolysis of the isopropyl ester of DL-phenylalanine.

The author wishes to thank Dr. William C. Rose for his helpful suggestions and for generously making available the facilities of his laboratory.

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THE EFFECT OF AMINO GUANIDINE ON THE OXIDATION OF FORMALDEHYDE BY RAT LIVER

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Formaldehyde was shown to be a normal metabolite when Handler, Bernheim, and Klein (1) found sarcosine oxidase which catalyzes the oxidative demethylation of sarcosine to glycine and formaldehyde. Sakami (2), Welsh and Sakami (3), and Stekol *et al.* (4) have recently presented evidence that formate may provide the animal with necessary methyl groups. It was therefore of interest to investigate the oxidation of formaldehyde to formic acid *in vitro* in animal tissue. It apparently has been assumed that xanthine oxidase or aldehyde oxidase is responsible for its metabolism.

EXPERIMENTAL

Rat liver was ground in a mortar with 0.05 M Na-K-phosphate buffer, 1.0 ml. per gm., and strained through muslin. 0.5 ml. of the resulting suspension was used in each Warburg vessel with a fluid volume of 2.0 ml. Washed preparations were made by centrifuging the suspension after diluting with water to 50 ml., discarding the suspension, and washing again with buffer and water.

When 0.1 mg. of formaldehyde was added to the unwashed suspension, the oxygen uptake was inhibited for 10 to 20 minutes. The inhibition then disappeared and the formaldehyde was apparently oxidized fairly rapidly to formate, which was then oxidized more slowly to carbon dioxide. When 0.2 mg. of formaldehyde was added, the same sequence occurred except that the inhibition was greater and more prolonged. With larger amounts the inhibition was not overcome and there was no apparent oxidation. When washed preparations were used, formaldehyde was not oxidized.

These facts suggested that formaldehyde first inhibited some oxidative enzymes from which it slowly dissociated to be oxidized to formate, or that it combined eventually with amino groups of soluble compounds and was oxidized when still combined. On the assumption that the latter mechanism was more likely, formaldehyde was mixed with a number of amino compounds before it was added to the liver suspension. These included amino acids, amines, ammonium salts, urea, hydrazine, phenylhydrazine, guanidine, and aminoguanidine. All were without effect except the last.

When aminoguanidine and formaldehyde were mixed and then added to the liver suspension, a rapid oxygen uptake occurred. Aminoguanidine itself was without effect. The uptake was proportional to the concentration of formaldehyde and independent within limits of the concentration of aminoguanidine. The latter was not acting as a catalyst. As shown in Fig. 1, the uptake required approximately equimolar amounts of both substances to get maximum effects. If too little aminoguanidine was present, some formaldehyde either remained uncombined or reacted with

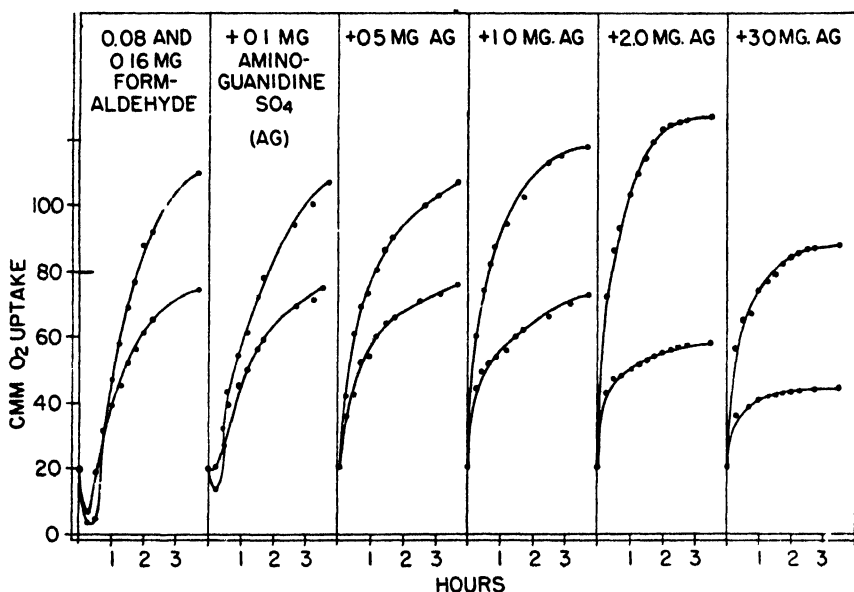


FIG. 1. The oxidation of two concentrations of formaldehyde with and without different concentrations of aminoguanidine sulfate by rat liver suspension at pH 7.8 and 37°. The control oxygen uptake of the liver has been subtracted in each case. The theoretical uptake for the oxidation of 0.08 mg. of formaldehyde to carbon dioxide is 60 cmm. of oxygen.

the other amino groups in the molecule. If too much was present, polymerization probably occurred, for both the rate and amount of oxygen uptake decreased. It thus appears that formaldehyde is oxidized when combined with aminoguanidine. In confirmation of this it is possible to mix equimolar amounts of the two, and evaporate to dryness; the resulting product, which is amorphous, was rapidly oxidized when added to liver with an uptake proportional to the amount added. If less than an equimolar amount of formaldehyde was used, the resulting product was oxidized slowly or not at all.

The amount of oxygen taken up shows that formaldehyde was oxidized to carbon dioxide and water. Formate, added to liver suspensions, was oxidized and the rate was not affected by aminoguanidine. If the suspension was washed, neither formaldehyde nor formate was any longer oxidized and the formaldehyde-aminoguanidine complex was oxidized to the formate stage. This is shown in Fig. 2. The inability of washed liver suspension to oxidize the formaldehyde may mean that a substance in liver with which formaldehyde can combine had been washed away or that

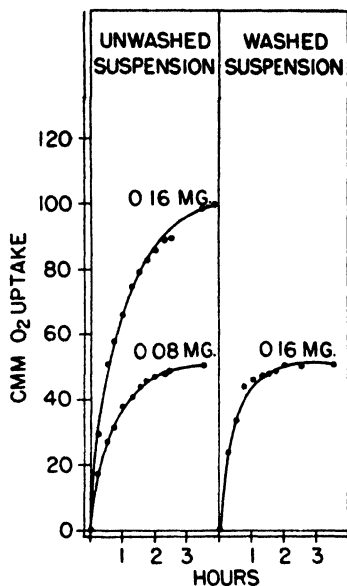


FIG. 2

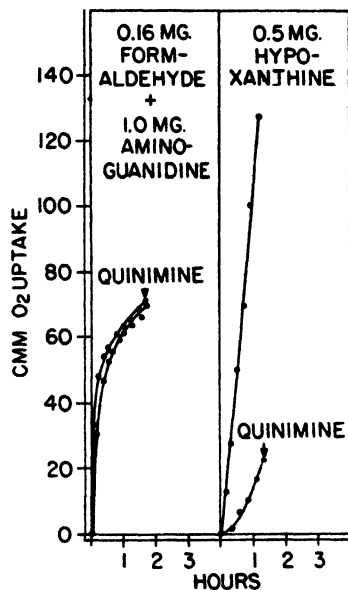


FIG. 3

FIG. 2. The oxidation of formaldehyde in the presence of 1.0 mg. of aminoguanidine sulfate with washed and unwashed suspensions at pH 7.8 and 37°.

FIG. 3. The effect of 2×10^{-4} M quinimine, added at the beginning, on the oxidation of the formaldehyde-aminoguanidine complex and on the oxidation of hypoxanthine by rat liver suspension at pH 7.8 and 37°.

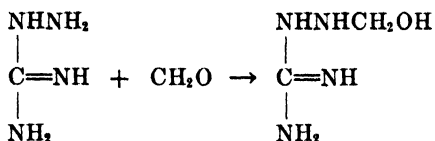
an enzyme which may oxidize it in the free state had been inactivated. In neither washed nor unwashed suspensions did aminoguanidine catalyze the oxidation of acetaldehyde or other straight chain and aromatic aldehydes. When formaldehyde was released from sarcosine by the action of the sarcosine oxidase, aminoguanidine, added to the suspension, combined with it and increased the oxygen uptake. Acetaldehyde produced by the action of the alcohol oxidase on ethyl alcohol is not affected under the same conditions.

Two experiments show that the xanthine oxidase is not responsible for

the oxidation of the aminoguanidine-formaldehyde complex. First, the milk enzyme which oxidizes formaldehyde does not oxidize the complex. Incidentally, this is a further indication that the complex does not dissociate to release formaldehyde. Secondly, quinimine, which inhibits the xanthine oxidase of rat liver (5), has no effect under the same conditions on the oxidation of the complex. This is shown in Fig. 3. The identity of the enzyme responsible for the oxidation has not been determined. Since it is not inhibited by 1×10^{-2} M cyanide, it cannot be either the alcohol or choline oxidases. It is not inhibited by relatively large amounts of guanidine, urea, or hydrazine, nor by amino acids. It is present in rat kidney in a lesser amount and absent from brain. It is absent from guinea pig and hamster liver and kidney. Its pH optimum is approximately 8.0; it is completely inactive at pH 6.0. Its activity is destroyed by heating for 2 minutes in a boiling water bath.

DISCUSSION

It is possible to write the reaction between aminoguanidine and formaldehyde as follows:



The compound resembles no known metabolite and it is not possible to attribute its oxidation to any recognized enzyme. On the basis of these results, however, it can be assumed that a similar reaction may take place between formaldehyde and a normal component of tissue.

SUMMARY

1. Formaldehyde is rapidly oxidized by rat liver if it is mixed in approximately equimolar amounts with aminoguanidine. Guanidine, amines, amino acids, hydrazine, and urea are without effect.
2. The properties of the enzyme catalyzing the oxidation are described.

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AMINO ACID REQUIREMENTS OF LACTOBACILLUS LEICHMANNII

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In the course of development of microbiological methods for the determination of amino acids and of vitamin B₁₂ (1), studies were initiated to determine the amino acid requirements of *Lactobacillus leichmannii* 313 (ATCC 7830)¹ and *Lactobacillus leichmannii* 327 (ATCC 4797), both widely used in studies on the specificity of their requirement for vitamin B₁₂ (cf. (2)). Detailed information on the nutritional requirements of these organisms is essential for the quantitative determination of amino acids in purified proteins and foods with *L. leichmannii*. The use of this organism would be valuable for amino acids for which adequate methods are not available or as a supplement to existing methods.

The data obtained on the qualitative and quantitative amino acid requirements, improvements in the composition of the basal medium, detailed studies on the utilization of cystine and cysteine, and the use of this organism for the determination of amino acids in proteins and foods are presented in this paper.

EXPERIMENTAL

Initially, tests were conducted with the basal medium (purified amino acids) described previously for amino acid studies with *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* P-60 (3). Additional supplements added per 100 ml. of medium were injectable liver extract² (0.1 ml.) as a source of vitamin B₁₂ and Tween 80 (0.2 ml.). A total volume of 2 ml. per tube was used and the acid production was determined after incubation at 37° for 72 hours. The tubes were inoculated after autoclaving at 15 pounds pressure for 10 minutes. The preparation of the stock cultures and liquid inocula of these organisms has been described (1).

With this procedure the growth and acid production were limited (titrations of 4.0 to 6.0 ml. of 0.02 N NaOH per tube). Factors found to increase the acid production equivalent to 18 to 20 ml. of 0.02 N NaOH

¹ The original stab cultures were obtained from Dr. E. E. Snell, University of Wisconsin.

² 15 U. S. P. units per ml.

were (1) replacement of cystine with cysteine, (2) doubling the concentration of all of the amino acids, and (3) increasing the concentration of liver extract in the liquid inoculum. The major improvement in the initial tests was obtained when the amounts of all of the amino acids in the medium were increased simultaneously with the replacement of cystine with cysteine. Subsequent tests revealed that the increase in the amount of cysteine (as the hydrochloride) from 0.4 mg. per tube to 0.8 mg. per tube was the major factor in improving the acid production. Similar titrations were obtained in tests conducted simultaneously with *L. leichmannii* 313. The basal medium for subsequent tests conducted on the qualitative and quantitative amino acid requirements of these organisms was therefore modified to include cysteine at 0.8 mg. per tube. Standard curves obtained with various amino acids were similar with the other amino acids either added in the usual amounts or increased by 100 per cent.

A summary of the results obtained on the qualitative amino acid requirements of *L. leichmannii* 313 and 327 is presented in Table I. Standard curves were obtained with graded levels of the test amino acid in each of the experiments performed. The range in the amino acid concentration used in the quantitative work is also presented. No essential differences were noted in the quantitative requirements of the two test organisms; consequently, the composite results only are given.

The qualitative amino acid requirements of the two strains were also found to be similar. It will be noted that strain 313 has a greater capacity to synthesize threonine than does strain 327 and the converse is true for serine and leucine. The data on the qualitative requirements reflect not only the actual requirements but also the amounts of individual amino acids contributed to the medium as impurities. These amino acids, however, have been used in the media for microbiological assays with *L. arabinosus*, *L. mesenteroides* P-60, and *Leuconostoc citrovorum* 8081 as the test organisms and the titration blanks, when the appropriate amino acid was omitted, are also indicated in Table I. It is apparent from these data that the high blanks obtained with *L. leichmannii* for several of the amino acids (leucine, lysine, threonine, tyrosine, proline, serine, and glycine) reflect the ability of these strains to synthesize these amino acids to some extent. Of considerable interest is the requirement of both *L. leichmannii* strains for alanine, which is not required by most lactic acid bacteria (4-6). Preliminary studies indicate that *L. leichmannii* will be useful in determining the alanine content of proteins.

The data on the quantitative amino acid requirements (Table I), with the exception of cystine, are similar to those obtained for the other test organisms used in this laboratory. The amount of cystine or cysteine required (100 to 500 γ per tube) was greatly in excess of the amount re-

quired by *L. mesenteroides* under similar testing conditions (20 γ per tube). Consequently, studies with the addition of graded levels of cysteine and cystine (up to 500 γ per tube), with combinations of cystine and cysteine or sodium hydrosulfite as a reducing agent, with glutathione, or with aseptic addition of the standards after autoclaving were conducted. The

TABLE I
Amino Acid Requirements of *L. leichmannii* 313 and *L. leichmannii* 327

Amino acid omitted	Blank titrations (ml. 0.02 N NaOH)*			Range, standard curve (γ per tube)
	<i>L. leichmannii</i> 313	<i>L. leichmannii</i> 327	Other organisms†	
None.	18.0	20.2		
Leucine..	3.4	5.0	1.4 (1)	0-40
Valine .	0.9	0.8	0.9 (1)	0-50
Isoleucine.	0.8	1.6	0.9 (1)	0-50
Lysine .	12.8	11.8	0.6 (2)	0-60
Arginine .	1.3	1.1	1.8 (3)	0-50
Histidine	1.9	1.8	1.5 (2)	0-16
Threonine	11.3	4.7	1.8 (3)	0-30
Phenylalanine	2.2	2.9	3.0 (1)	0-25
Tryptophan .	1.7	1.4	1.0 (3)	0-2
Methionine	1.8	2.2	0.6 (2)	0-20
Glutamic acid‡.	1.9	1.4	4.2 (1)	0-100
Aspartic " ..	0.9	1.6	0.2 (2)	0-100
Tyrosine.. . . .	7.8	7.3	3.8 (2)	0-24
Proline ..	17.5	20.2	0.3 (2)	
Serine . . .	12.9	16.6	10.0 (2)	
Alanine ..	2.2	2.7	2.0 (4)	0-30
Glycine ..	18.1	19.9	2.2 (2)	
Cystine ..	1.7	1.7	1.7 (2)	0-300 (see text)

* The titration value for uninoculated tubes has been subtracted from the blank values.

† (1) *L. arabinosus*, (2) *L. mesenteroides*, (3) *S. faecalis* R, and (4) *L. citrovorum*.

‡ Aspartic acid replaced by asparagine in the medium for *L. leichmannii*; for *L. arabinosus*, 50 γ of glutamine added per tube prior to autoclaving.

majority of these studies were conducted with strain 327, although comparable results were obtained with strain 313.

When cystine was added in graded amounts, the acid production by the test organisms varied widely in repeated experiments. The maximum acid production ranged from a titration of 8.0 ml. to 19.5 ml. Cystine was usually as active as cysteine (free base) in promoting acid production when less than 100 γ per tube was added. In many experiments apparent inhibition of acid production occurred with high levels of cystine (300 γ or

more per tube) but never with cysteine. For example, when 100 γ of cystine were added per tube, an average titration of 19.5 ml. was obtained, while the titrations when 200, 300, 400, or 500 γ per tube were added were 16.8, 11.4, 5.6, and 3.1 ml. respectively. A less marked inhibition was observed when tests were carried out with a total volume of 10 ml. per tube. The inhibition could be counteracted to a limited extent by the addition of cysteine or sodium hydrosulfite.

In an attempt to resolve these observations, subsequent experiments were carried out to determine the stability of cystine and cysteine during autoclaving and to determine the activity of glutathione. The results obtained in representative experiments are presented in Figs. 1 and 2.

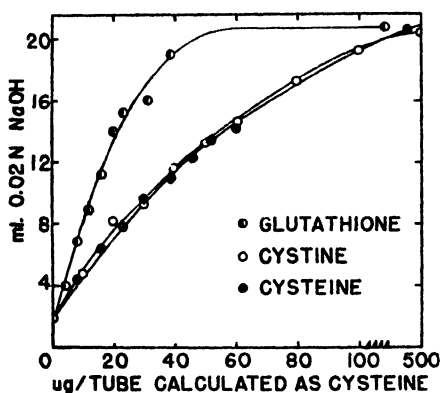


FIG. 1

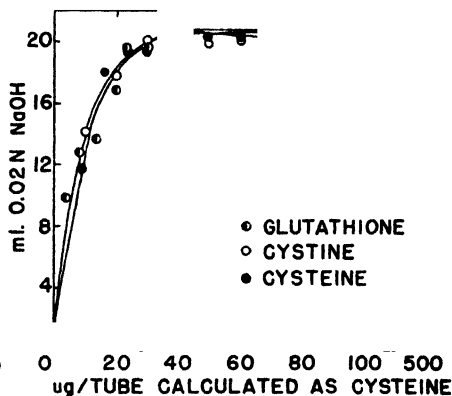


FIG. 2

FIG. 1. Titration curves obtained when graded levels of cysteine, cystine, and glutathione were added to the tubes prior to autoclaving. *L. leichmannii* 327 was used as the test organism.

FIG. 2. Titration curves obtained when graded levels of cysteine, cystine, or glutathione were added aseptically to the tubes after autoclaving separately from the medium. *L. leichmannii* 327 was used as the test organism.

It will be noted that glutathione, when autoclaved with the medium, was much more active on a molar basis than cysteine or cystine, but was equal in activity to cystine or cysteine when added aseptically after sterilization. Autoclaving inactivates more cystine or cysteine (80 to 90 per cent) than glutathione (50 per cent). These and other data (7) show that the activity of cystine, cysteine, or of cysteine as glutathione, and related compounds varies considerably for different test organisms. The marked differences in the amounts of cystine or cysteine required for maximum growth of *L. leichmannii* as compared to *L. mesenteroides* are of the same magnitude as the differences for *L. casei* as compared to *L. mesenteroides* (7). In these particular tests no inhibition occurred when 500 γ of cystine per tube were added prior to autoclaving, but an inhibition was observed

when the cystine was sterilized separately and added aseptically. These results are similar to data reported for *L. casei* (7).

Since these organisms require glutamic acid (Table I), the glutamic acid activity of glutathione was also studied. For these tests, asparagine was added to the basal medium in place of aspartic acid. On a molar basis, glutathione added before autoclaving was 70 per cent as active as glutamic acid, but, when the glutathione was added aseptically, it was 90 to 100 per cent as active. Glutamic acid was equally active when added to the medium before and after autoclaving. The glutamic acid activity of glutathione noted here approximates that observed in studies with other lactic acid bacteria, and the utilization of amino acids from this naturally occurring peptide by lactic acid bacteria is greater than observed for most synthetic peptides tested (5, 8, 9).

TABLE II

Amino Acid Composition of Certain Natural Products Determined with Different Test Organisms

All the values are calculated as per cent of partially dried and defatted samples.

Test organism*	Methionine			Histidine			Arginine			Nitrogen
	1	2	3	1	2	3, 4	1	2	4	
Casein ..	2.71	2.95	2.65	2.93	2.78	2.90	3.69	3.44	3.66	15.10
Crystalline bovine plasma albumin†	1.10	0.83	0.99	3.67	3.30	3.58	5.71	5.74	6.15	15.70
Lamb leg	2.13	2.34	2.11	2.48	2.36	2.15	5.83	6.05	5.76	13.51
" breast	2.11	2.23	1.95	2.40	2.16	2.10	5.78	6.00	5.64	13.42
" leg (cooked)	2.37	2.49	2.12	2.74	2.44	2.41	6.73	6.90	6.45	14.40

* 1, *L. leichmannii* 327; 2, *L. leichmannii* 313; 3, *L. mesenteroides* P-60; 4, *S. faecalis* R.

† We are indebted to the Armour Laboratories for supplying this preparation.

These studies were then extended to the use of *L. leichmannii* 313 and 327 as test organisms for the determination of amino acids in proteins and foods. These tests were conducted with 0.8 mg. of cysteine hydrochloride added per tube. The results obtained for methionine, histidine, and arginine are presented in Table II. The values have been compared with those obtained by other microbiological methods that have been used for several years. The methods used for preparation of the samples have been described (3).

The results obtained were in fair agreement for the different samples assayed with three or more test organisms. Several of the values obtained for the meat samples with *L. leichmannii* were somewhat higher than those obtained with the other lactic acid test organisms. We have used *L. leichmannii* for the analysis of several amino acids. The reproducibility of the data obtained within a single assay and for repeated assays was

somewhat less satisfactory than for the other test organisms used. *L. leichmannii* is useful, however, as a test organism for evaluating the validity of existing methods for the determination of amino acids but may be even more useful for determining alanine, an amino acid for which only *L. citrovorum* 8081 has been proposed as the assay organism (10).

SUMMARY

Lactobacillus leichmannii 313 and 327 do not require proline or glycine in the medium, can synthesize serine, threonine, lysine, tyrosine, and leucine to some extent, and both strains require alanine.

The amounts of each of the amino acids required for maximum growth were similar to those required by *Lactobacillus arabinosus*, *Streptococcus faecalis* R, *Leuconostoc mesenteroides* P-60 or *Leuconostoc citrovorum* with the exception of cystine. The response to graded levels of cystine or cysteine was variable in repeated experiments. From 100 to 500 γ per tube (2 ml., total volume) were required for maximum growth, and in some experiments additions of cystine in excess of 200 γ per tube resulted in an inhibition of acid production.

Glutathione-cysteine was much more active than cystine or cysteine when the additions were made prior to autoclaving, but the three compounds were equal in activity (calculated as cysteine) when sterilized separately from the medium. The glutamic acid activity of glutathione was 70 per cent of that of free glutamic acid when the additions were sterilized in the presence of the medium and 100 per cent when sterilized separately.

The use of *L. leichmannii* 313 and *L. leichmannii* 327 as test organisms for the quantitative determination of amino acids in purified proteins and foods was investigated. Representative data obtained for methionine, arginine, and histidine are presented and compared with data obtained with other test organisms.

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ISOLATION OF COENZYME A*

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A preliminary account of the chemistry of coenzyme A (Co A) has been published previously (1). Since the definite purification of the coenzyme has met so far with considerable difficulty, an account is given here of the isolation and analysis of our most highly purified preparation. This preparation, however, is not likely to represent pure Co A.

Isolation of Coenzyme A

The coenzyme content was followed throughout the purification by the assay method described in a preceding paper (2). Hog liver was found most suitable. Ten 50 pound batches of liver were used in this preparation. Since rapid autolysis of Co A occurred, the yield was largely dependent on the rapidity with which the livers were obtained. Livers from still warm hog bodies were cut into small portions and added to an equal volume of boiling water. The temperature was not allowed to drop below 80°. After completion, the mixture was kept simmering for 15 minutes more. The juice was passed through muslin.

Deproteinization and Mercury Precipitation—The juice was cooled and deproteinized with 5 per cent trichloroacetic acid. The filtrate was, as quickly as possible, neutralized to pH 5 and the coenzyme precipitated with a slight excess of mercuric acetate. The precipitate was collected after settling overnight, and decomposed with hydrogen sulfide.

Acetone Fractionation and Barium Precipitation—The aerated filtrate from mercuric sulfide was neutralized to pH 4 to 5; 2 volumes of acetone were added, and the precipitate discarded. Another 7 volumes of acetone were added, and, after being kept overnight in the cold, the oily yellow deposit was collected by decantation. The oily deposit was dissolved in water, neutralized to pH 8.4 to 9, and barium acetate was added, followed by 2 volumes of acetone. The precipitate was dried with alcohol and ether. 50 pounds of liver gave 35 gm. of barium salt of 1.5 units per mg.

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Lead, Silver, and Second Barium Treatment—The barium salt was dissolved by addition of *N* acetic acid to about pH 4, a dirty residue was discarded, and a slight excess of lead acetate was added. The lead precipitate was decomposed with hydrogen sulfide and the lead sulfide reextracted with addition of sodium acetate to bring it to pH 4.5. The combined filtrates were brought to pH 4.5 and silver nitrate was added. The silver was removed with hydrogen sulfide and the filtrate brought to pH 8.5 to 9. Barium acetate was added, followed by half the total volume of alcohol. The precipitate was collected and dried with alcohol and ether. From about 100 pounds of liver were obtained 1.5 gm. of this barium salt of 30 units per mg.

Coprecipitation with Barium Sulfate and Elution; Phosphotungstic Acid Precipitation—The barium salt was dissolved to make a 1 per cent solution with *N* acetic acid and a small residue discarded. For every 100 ml., 6 ml. of 6 per cent barium acetate were added and 6 ml. of 0.2 *M* sodium sulfate dropped slowly in with agitation. Barium sulfate was thus precipitated in the presence of a considerable excess of barium ion. The precipitate was collected and this procedure repeated three times. The combined barium sulfate precipitates were extracted with 0.2 *M* sodium sulfate in 0.1 *N* acetic acid. Approximately 25 ml. were used per gm. of original barium salt. The extraction was carried out at 50° with vigorous stirring, and repeated three times. The combined eluates were cooled, the solution made 0.8 *N* with concentrated hydrochloric acid, and an excess of phosphotungstic acid was added. The precipitate was washed carefully three times with acidified acetone and dried with ether. The activity of this compound averaged close to 90 units per mg.

Third Barium Treatment and Precipitation with Acetone-HCl—A 1 per cent solution of the compound was brought to pH 8.5, and the Co A precipitated with barium acetate and an equal volume of alcohol. 850 mg. of barium salt were obtained with an activity of 110 units per mg. The barium salt was dissolved with a comfortable excess of hydrochloric acid, and 5 ml. of *N* sulfuric acid were added. After removal of barium sulfate, 9 volumes of cold acetone were added, and the white precipitate was dried with acetone and ether. This preparation was whitish.

The total yield from 500 pounds of liver was 625 mg., having an activity of 130 units per mg. It was through this compound that we were able to establish pantothenic acid as a part of the coenzyme and to obtain preliminary results on its constitution. This preparation will now be referred to as Preparation A. All analyses reported in this paper are for this preparation.

Analysis of Preparation A

Pantothenic Acid Content—When it became apparent that the coenzyme could not be replaced in its function by any known coenzyme, it was presumed that we were dealing with a new factor. Accordingly, to elucidate its nature further, the preparation was assayed for the presence of any vitamins. Table I shows the content of various B vitamins in this preparation. The B vitamins, including pantothenic acid, appeared to be present in only negligible amounts. It was noticed, however, that the pantothenic acid value by test with *Lactobacillus arabinosus* increased slightly on digesting with clarase and papain (3).

This observation suggested the possibility that the enzymatic treatment with clarase and papain, as used in the routine assay for pantothenic acid

TABLE I
Vitamin Content of Preparation A

Vitamins	Per cent
Nicotinic acid.	0.06
Folic acid.	0.0002
Riboflavin.	0.006
Inositol.	0.05
Pyridoxine.	0.03
Biotin, thiamine	Not detectable
Pantothenic acid	
Direct.	0.085
1 wk. incubated with papain-clarase	0.16
From β -alanine, after acid hydrolysis	11.0

(3), might liberate only very slowly the pantothenic acid from Co A (cf. (4)). Therefore, the sample was subjected to acid hydrolysis and then tested for the presence of β -alanine, with yeast as the test organism. The yeast assay revealed then the presence of amounts of β -alanine in Co A corresponding to a pantothenic acid content of 11 per cent. Additional tests for β -alanine in Co A samples of varying degrees of purity confirmed the presence of an unusually large amount of β -alanine.

The procedure which eventually permitted us to show by direct microbiological assay that Co A is a pantothenic acid derivative was the use of two enzymes which had previously been shown to inactivate Co A (5). These were intestinal phosphatase and an unidentified enzyme present in extracts of acetone pigeon liver powder. The combined action of these two enzymes releases 85 to 90 per cent of the pantothenic acid calculated from the β -alanine content. A detailed account of this enzymatic method

of pantothenic acid liberation appeared in a previous paper from this laboratory (4).

A large series of Co A preparations, ranging in potency from 9 to 132 units per mg., was subjected to enzymatic treatment and the pantothenic acid was determined microbiologically by the method of Skeggs and Wright (6). Table II illustrates the constancy of the amount of pantothenic acid per unit of activity, regardless of the degree of purity of the preparation.

TABLE II
Unit Activity of Coenzyme A (2) Compared with Pantothenic Acid Content

Preparation No.	Activity	Pantothenic acid	β -Alanine	Micrograms pantothenate Unit activity	
				From panto- thenic acid	From β -alanine
	units per mg.	γ per mg.			
105	9	7.0	5.0	0.78	1.35*
100	10	10.0	4.6	1.0	1.13*
PD	14	9.3		0.66	
137	14	8.0		0.57	
205	20	14.3		0.72	
72	26	18.0	9.0	0.69	0.86
101	32	18.0	9.0	0.57	0.73
133	35	22.5		0.64	
102	60	37.0	19.1	0.62	0.78
102a	60	42.5		0.71	
100a	60	42.0		0.70	
99	65	44.0	21.2	0.68	0.84
LC	100	70.0		0.70	
103	100	65.0	29.6	0.65	0.73
A	130	93.0	46	0.72	0.86
A ¹	132	84.0	45	0.64	0.86
Average....				0.69	0.81

* Omitted from the average.

Adenine—Spectrophotometric analysis of the coenzyme in the ultra-violet gives an absorption curve which is indicative of adenine. After nicotinic acid which absorbs, although less strongly, at 260 $m\mu$ was excluded, a single symmetrical maximum at 260 $m\mu$, which showed practically no shift with the change of pH, indicated adenine to be the only substance present in appreciable quantity which absorbs in the ultraviolet. From the absorption at 260 $m\mu$, the adenine content for Preparation A was estimated to be 20 per cent.

For chemical identification, 30 mg. of Preparation A were hydrolyzed

for 60 minutes in N HCl . The adenine was isolated from the hydrolysate as the picrate, following the procedure of Warburg and Christian (7). The yield was 16.1 per cent, or 81 per cent of the spectrophotometric value. Warburg and Christian obtained from triphosphopyridine nucleotide about 80 per cent of the theory with a melting point (with decomposition) of 285° . We treated a sample of yeast adenylic acid similarly and obtained 86.5 per cent recovery. The chemical and spectrophotometric analyses were thus in good agreement. The picrates isolated both from Preparation A and from our sample of adenylic acid crystallized in clusters of fine needles of identical appearance (*cf.* (8) for the different crystallization patterns of adenine picrate). The melting point (with decomposition) (slow heating, uncorrected) of the picrate from Compound A was 283° , and from our adenylic acid, 282° . The mixed melting point (with decomposition) was 284° .

Ribose—The ribose content of this preparation as determined by the orcinol method (9) was 22.4 per cent.

There is very little direct reducing sugar in the compound as determined by the colorimetric ferricyanide method (10). On hydrolysis with 0.1 N hydrochloric acid at 100° for 15 minutes, a value of 26.5 per cent reducing sugar (as pentose) is obtained by this procedure. Hydrolysis with 0.1 N sodium hydroxide at 100° for 15 minutes gives no increase in reducing sugar. Since reducing sugar appears only on treatment with acid, it is assumed that the ribose in the coenzyme preparation is bound to adenine.

Phosphorus—The total phosphorus content of Preparation A is 9.1 per cent. This corresponds to a ratio of 2 moles of phosphate for every mole of adenine, and approximately 6 moles of phosphate for every mole of pantothenic acid. The preparation does not contain any free phosphate. In Fig. 1, the hydrolysis curve of Preparation A is presented in N hydrochloric acid. Approximately 28 per cent of the total phosphate is liberated in 7 minutes and 46 per cent in 30 minutes.

Nature of Adenylic Acid—The analytical data summarized in Table III indicate that Preparation A contains 50 per cent adenylic acid. Furthermore, for every mole of adenosine there are exactly 2 moles of phosphoric acid present. Attempts to identify 5-adenylic acid have failed so far. Tests with Schmidt's muscle deaminase were carried out after treatment with a potato nucleotidase (11), which splits the pyrophosphate link in coenzyme I and flavin adenine dinucleotide. In experiments to be reported in a subsequent paper, it is shown that this enzyme also splits Co A without liberation of inorganic phosphate. However, no 5-adenylic acid seems to appear after this treatment.¹ The intact Preparation A was

¹ We are indebted to Dr. Sidney Colowick for carrying out these tests.

tested with adenylic acid-dependent muscle phosphorylase without showing any effect.² These tests seem to have to be amplified before definite conclusions can be drawn.

TABLE III
Analytical Data for Preparation A

	Per cent	Ratio
Pantothenic acid	11.0	1
Adenine, ultraviolet at 260 m μ	20.0	3
" isolated as picrate	16.1	
Ribose, colorimetric	22.4	3
Phosphorus	9.1	6
Nitrogen (Kjeldahl, Dumas)	11.9	17
Sulfur	1.97	1.2
Cystine (microbiologically)	6.1	1

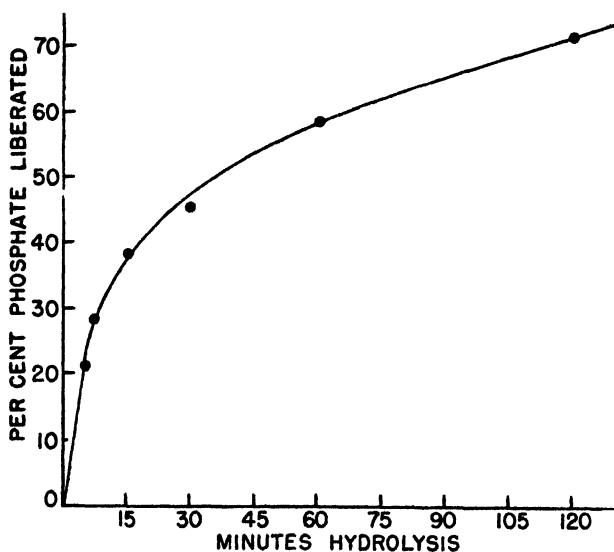


Fig. 1. Hydrolysis of Preparation A in normal hydrochloric acid of 100°

The acid hydrolysis curve, shown in Fig. 1, may be taken to indicate at least two phosphate links of different stability. The hydrolysis curve with *N* hydrochloric acid shows a quite distinct break; just about 50 per cent of the phosphate is liberated after 30 minutes hydrolysis. From then on the hydrolysis proceeds at a much slower rate. Since much of

² Dr. Gerty Cori very kindly made these tests.

the adenylic acid most likely is a contaminant, any rational interpretation appears rather difficult at the present time.

In any case, the data on pantothenic acid liberation from Co A by intestinal phosphatase (4) make it quite likely that part of the phosphate must be linked to pantothenic acid, presumably through one of the hydroxyl groups.

Cystine—It was observed that, during alkaline hydrolysis of Preparation A, considerable amounts of hydrogen sulfide were produced. The preparation gave, however, no direct nitroprusside test. On treatment with sodium cyanide, a strongly positive test was obtained, indicating the presence of cystine. Sulfur determination³ showed a 1.97 per cent content which would correspond to 7.5 per cent cystine. Glutathione was excluded through a negative methylglyoxalase test. In the hydrolysate 6.1 per cent cystine was determined microbiologically; no methionine was found. The cystine content of the present preparation is of a magnitude similar to that of pantothenic acid.

Glutamic Acid—Tests for glutamic acid were not carried out with Preparation A, but with a similar compound of 100 units per mg. of activity. With squash decarboxylase (12), 4 per cent glutamic acid was determined in the hydrolysate. The preparation contained 6.8 per cent pantothenic acid, or approximately 1 mole of glutamic acid per mole of pantothenic acid. The presence of glutamic acid in a degradation product of Co A was first observed by Cheldelin and his group (13).

DISCUSSION

Procedure—The method of isolation described here is quite involved. It gave the best preparation which we have obtained so far. Efforts are in progress to simplify the procedure and some headway is being made with the use of charcoal adsorption, while promising results have been obtained with counter-current distribution.

Chemistry—The chemical analysis reported here and previously reported (4, 14) observations on enzymatic inactivation and degradation invite some cautious mapping of the structure of Co A. Pantothenic acid obviously is linked through a phosphate, possibly a pyrophosphate bridge, to another molecule, presumably adenylic acid. This is indicated by the inactivation and attendant partial liberation of pantothenic acid through intestinal phosphatase, acting as a phosphodiesterase. The need of a second enzyme to liberate the vitamin fully indicates a second link to pantothenic acid. This second link, split by the liver enzyme, remains

³ For the sulfur analysis we are greatly indebted to Dr. Vincent du Vigneaud.

to be determined. No definite information on the nature of this link has emerged so far.

The pantothenic acid structure (Fig. 2) offers essentially three points of attachment; namely, two hydroxyl groups and one carboxyl. Phosphate would most likely attach to one of the hydroxyls. The presence of amino acids in our preparations invites speculation on a peptide link to the carboxyl, this being possibly the link split by the liver enzyme. There are, furthermore, strong indications that Co A-enzyme complexes bind acetyl groups, as, for example, by reaction with acetyl phosphate (15, 16). Indication also has appeared that Co A may act as a phosphate carrier (15). The present data obviously do not allow more than a rather preliminary formulation to account for chemical and functional data on Co A.

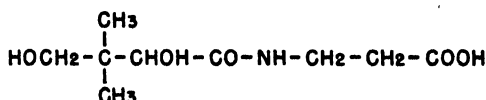


FIG. 2. Pantothenic acid

SUMMARY

The isolation of Co A from hog liver is described. The procedure outlined in this paper yielded the most potent preparation so far obtained. This preparation assayed 130 units per mg. It contained 11.3 per cent pantothenic acid, 9.1 per cent phosphorus, 20 per cent adenine, 22.4 per cent ribose, 6 per cent cystine, and probably some glutamic acid. Evidence is obtained indicating that the coenzyme is composed of three major components: pantothenic acid, Component 1 being linked (a) through a phosphate bridge to Component 2, presumably adenylic acid, and (b) through an as yet undetermined link to Component 3, possibly an amino acid. The nature of the 50 per cent adenylic acid contained in this preparation remains to be determined.

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EFFECT OF HORMONES UPON THE PRODUCTION OF KETONE BODIES BY RAT LIVER SLICES

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(Received for publication, February 17, 1950)

Numerous investigators have established the important rôle played by the endocrine glands in the regulation of fat metabolism in the intact animal. The administration to fasting rats of anterior pituitary growth hormone and adrenocorticotrophic hormone (ACTH) (1) has been shown to increase both the concentration of ketone bodies in the blood and the rate of their excretion. Adrenalectomy, on the other hand, tends to have the opposite effect (2). Since these effects are believed to be due to alterations of the rate of ketone body production by the liver (1), it appeared desirable to investigate the effects of these endocrines upon the ketone body metabolism of surviving liver slices.

Methods

Male Sprague-Dawley rats, weighing 70 to 250 gm., were anesthetized with nembutal, and the livers were excised and immediately chilled in Petri dishes set in ice and lined with filter paper dampened with the incubation medium. Slices approximately 0.3 mm. thick were cut free-hand, weighed on a torsion balance, and 100 ± 5 mg. (wet weight) were distributed in duplicate 25 ml. Erlenmeyer flasks containing 2.0 ml. of Krebs phosphate-buffered medium at pH 7.4. The dry weight of similar samples of liver slices was determined for each animal, and all values were referred to this initial dry weight. The flasks were incubated in an atmosphere of 100 per cent oxygen in a Warburg water bath at 37°, with constant shaking at a rate of 120 strokes per minute, for periods of 5 to 120 minutes. After the incubation period, the contents of the flasks were treated with zinc sulfate, followed by barium hydroxide, to precipitate the proteins. The material was then decanted into a centrifuge tube. After centrifugation, duplicate aliquots of the supernatant were taken for the determination of ketone bodies by the method of Greenberg and Lester (3). Ketone body determinations were also made by the same method with blood from the tail vein or inferior vena cava. All the results given are the means of

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duplicate determinations on each of two flasks for each point, and represent the total ketone bodies (acetone plus acetoacetic acid plus β -hydroxybutyric acid) expressed as acetoacetate. In our hands, this method has had a standard deviation of 2.8 per cent, permitting the detection of differences of 7.7 per cent in sets of duplicate determinations, with a probability of less than 5 per cent.

The hypophysectomized rats used in this study were obtained from a commercial source.¹ They were observed over a period of 3 to 4 months before being used. Animals which grew appreciably during this time were not used in the study; however, animals which failed to grow but still maintained normal testicular size were used. A comparison of such "partially hypophysectomized" animals with totally hypophysectomized rats failed to show any differences of ketone body formation *in vitro*.

Animals were adrenalectomized in a single operation by the lumbar route. They were maintained on saline drinking water, and were used within 2 weeks after adrenalectomy. A careful search in each case failed to reveal adrenal tissue after death.

Thyroidectomy was performed by the usual technique, and the rats were maintained on calcium gluconate-calcium chloride drinking water for at least 2 weeks after the operation. Animals which continued to gain weight at a rate of 1 gm. or more per day were excluded as being incompletely thyroidectomized.

The hormones administered included crystalline growth hormone (4), adrenocorticotrophic hormone,² and thyroxine.³ Growth hormone was given in a dosage of 5 mg. per 100 gm. intraperitoneally 2 hours before the rats were killed. ACTH was given intraperitoneally in a dosage of 5.0 mg. per 100 gm. 3 hours before the animals were killed. Thyroxine was given subcutaneously in a dosage of 20 γ per day for 7 days.

Results

It is of interest to compare the metabolic activity of tissues prepared by us with the results obtained by others. The oxygen uptake of these preparations was $8.9 \pm 0.9 \mu\text{l. per mg. (dry weight) per hour}$, as compared with Edson's value of $10.5 \pm 1.9 \mu\text{l. per mg. (dry weight) per hour}$ (5). The rate of ketone body production over a 2 hour incubation period was $4.4 \pm 0.22 \mu\text{M of acetoacetate per 100 mg. (dry weight) per hour}$. This may be compared with the rates of $17.8 \mu\text{M per 100 mg. per hour}$ obtained by Cohen (6), $7.5 \pm 0.45 \mu\text{M per 100 mg. per hour}$ by Edson (5), and $4.0 \pm$

¹ Hormone Assay, Inc., Chicago, Illinois.

² Armour, lot 41-L-3, supplied through the courtesy of Dr. John R. Mote.

³ Supplied through the kindness of Dr. W. T. Salter, Department of Pharmacology, Yale University.

0.2 μM per 100 mg. per hour by Shipley (7) under comparable conditions. These values may not be an accurate measure of the rate of production of ketone bodies, because they are not corrected for the amounts of these substances present in the slices before incubation. In an effort to eliminate this potential error, the time course of formation of ketone bodies was studied and found to be constant from 5 minutes to 2 hours (Fig. 1). The lines representing the concentration of acetoacetate in the medium, extended back to zero time, intercept the ordinate at a positive value. This represents the preformed acetoacetate which diffuses rapidly from the slice into the medium, reaching equilibrium before 5 minutes. In these experiments, therefore, an initial observation of the ketone body

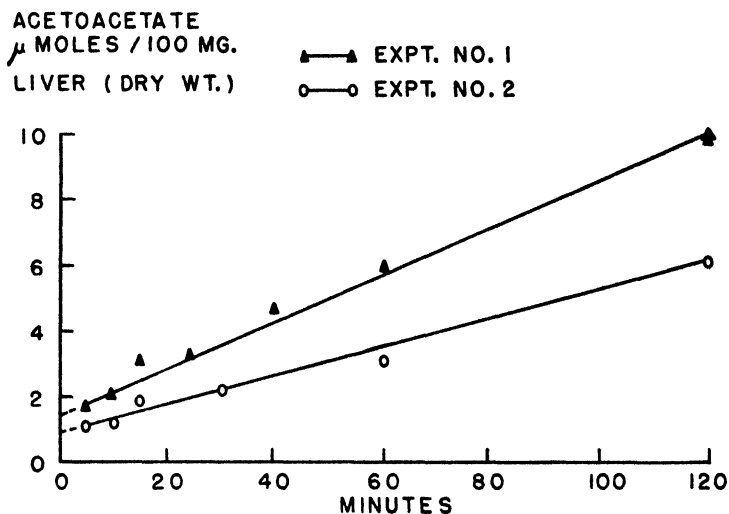


FIG. 1. Rate of formation of ketone bodies by rat liver slices

concentration in the system was made after 15 minutes incubation, and a final observation after 120 minutes. The difference between the two is taken as a measure of the true rate of synthesis of ketone bodies by the liver slices. The second point was chosen at 120 minutes because the curves show no tendency to level off up to that time. It is thus possible to eliminate exhaustion of substrate or of oxygen as limiting factors in determining the final rates. The rate of ketone body production thus determined is expressed as micromoles of acetoacetate produced per hour per 100 mg. of dry tissue (Q_{acac}).

The first series of animals studied was fasted for 18 hours before removal of the liver for slicing (Table I). Whereas hypophysectomy and thyroidectomy caused a highly significant depression of the Q_{acac} , adrenalectomy

caused a moderate but significant rise in the rate of production of acetoacetate. The effects of hypophysectomy can be explained entirely by the depression of thyroid function. The administration of maintenance doses of thyroxine to hypophysectomized animals restored their Q_{acac} to normal. No differences could be demonstrated in the blood ketone body concentration of normal, adrenalectomized, or hypophysectomized animals fasted for 18 hours (Table II).

Since we were unable to show a difference between adrenalectomized and normal rats either in the rate of formation of ketone bodies by liver

TABLE I

Effect of Various Endocrine Manipulations on Rate of Synthesis of Ketone Bodies by Surviving Rat Liver Slices in Krebs Phosphate Buffer after 18 Hour Fast

Incubation period 15 and 120 minutes at 37°; 100 ± 5 mg. of liver slices (wet weight) in 2 ml. of Krebs phosphate buffer. Gaseous phase 100 per cent oxygen. Expressed as micromoles of acetoacetate per 100 mg. of liver (dry weight) per hour for the increment from 15 to 120 minutes.

Treatment	No. of rats	Mean ± s.e.*	Compared to normal	
			t†	P‡
Normal.....	15	3.4 ± 0.35		
Hypophysectomized.....	7	1.3 ± 0.17	3.94	<0.01
Thyroidectomized.....	7	1.5 ± 0.25	3.49	<0.01
Adrenalectomized.....	6	4.7 ± 0.28	2.21	0.02 < 0.05
Hypophysectomized + thyroxine.....	3	3.4 ± 0.25		=1.00
Hypophysectomized + 1.5 mg. growth hormone daily for 2 days.....	2	1.2		

* S.e., $\sqrt{(\Sigma d^2)/(n(n-1))}$.

† Student's formula, corrected for small numbers.

‡ From Fisher's tables.

slices, or in the blood ketone body concentration after an 18 hour fast, the experiment was repeated with animals fasted for 40 hours, in the hope that this might bring out differences not discernible after the shorter period of deprivation. The data are presented in Table III. The Q_{acac} of normal rats was unchanged by prolonging the period of fasting. The rate appears slightly lower than normal in the livers of adrenalectomized rats, but the difference is not significant. The prolonged fast has, however, altered the Q_{acac} of the adrenalectomized rat liver, for the differences between the rats fasted 18 hours and 40 hours are highly significant ($P < 0.01$).

Although removal of the adrenal gland does not alter the Q_{acac} , and the

removal of the pituitary appears to exert its effect entirely through a reduction of thyroid activity, it seemed possible that effects might be

TABLE II
Effect of Various Endocrine Manipulations on Blood Ketone Body Concentration

Treatment	Blood ketone body formation after				18 hr. vs. 40 hr.		
	No. of rats	18 hr. fast	No. of rats	40 hr. fast	t°	P°	
		mg. per 100 ml.*		mg. per 100 ml.*			
		Concentration before treatment	Inter-val	Concentration after treatment	Change	t	P
		mg. per 100 ml.	hrs.	mg. per 100 ml.			
Normal.....	6	17.4 ± 2.3	16	47.4 ± 3.2	7.54	<0.01 >0.50	
Adrenalectomized.	4	15.2 ± 1.1	8	18.4 ± 2.7	0.26		
Hypophysectomized.....	3	15.4 ± 0.4					
Normal, 40 hr. fast, + 5 mg. growth hormone per 100 gm.....	4	49.1 ± 6.3	2	79.2 ± 5.4	30.1 ± 4.4	6.86	<0.01
Normal, 40 hr. fast, + 5 mg. ACTH per 100 gm.....	4	35.5 ± 4.2	3	44.2 ± 6.4	8.7 ± 3.1	2.83	0.1 > 0.05

* See foot-note, Table I.

TABLE III
Effect of Various Endocrine Manipulations on Rate of Synthesis of Ketone Bodies by Surviving Rat Liver Slices in Krebs Phosphate Buffer, 40 Hour Fast Techniques as outlined in Table I.

Treatment	No. of rats	Mean ± s.e.*	Compared to normal	
			t°	P°
Normal.....	8	3.4 ± 0.23	1.90	0.1 > 0.05
Adrenalectomized.	8	2.8 ± 0.30		
Normal + 5 mg. growth hormone per 100 gm.....	4	3.3 ± 0.11	0.59	>0.5
Normal + 5 mg. ACTH per 100 gm...	4	3.8 ± 0.38	0.72	>0.4

* See foot-note, Table I.

obtained by the administration of large amounts of growth and adrenocorticotrophic hormones. Experiments were therefore performed upon ani-

mals fasted 40 hours, following the injection of adrenocorticotrophic and of crystalline growth hormone. The administration of 5 mg. of these hormones per 100 gm. of body weight failed to alter the Q_{acac} in any way (Table III).

Both ACTH and growth hormone have a considerable effect upon the blood concentration of ketone bodies. Fasting from 18 to 40 hours causes a marked increase in the concentration of acetoacetate in the blood. Adrenalectomy prevents this increase (Table II). The administration of amounts of growth hormone and of ACTH which do not alter the Q_{acac} causes a significant increase in the blood ketone bodies (Table II). Growth hormone may be somewhat more effective than ACTH in this respect, since the increase following the administration of the latter is barely outside of the 5 per cent limits of probability.

In a few experiments it was found that growth hormones had no effect on ketone body production when incubated with liver slices from normal, 18 hour fasted hypophysectomized, 40 hour fasted hypophysectomized, and 40 hour fasted adrenalectomized animals.

DISCUSSION

These results are difficult to reconcile with previous observations of the effects of pituitary extracts *in vitro* and *in vivo* (1, 7, 8). Bennett *et al.* (1) have produced convincing evidence that one of the effects of growth hormone and of ACTH is to increase the production of ketone bodies in the intact animal. In the present experiments *in vitro*, however, the liver failed to respond as expected from the experiments on intact animals. Yet the potency of the hormone preparations and the accuracy of timing of the effects is attested by the fact that elevations of the blood ketone body concentration were produced both with growth hormone and with ACTH.

It is also difficult to explain the discrepancy between our results and those of Campbell and Davidson (8). It should be noted, however, that the increased Q_{acac} which they observed occurred 24 hours after the administration of a crude pituitary extract, whereas the present experiments tested the acute effects of highly purified hormone preparations, which were essentially free from extraneous physiological activity.

Shipley *et al.* (7) have reported an augmentation of the Q_{acac} after the addition of relatively crude pituitary extracts to liver slices incubated in rat serum. In their experiments smaller but definite effects were observed after the addition of boiled extract. This raises the possibility that they were observing an effect of some fraction other than those usually included among the pituitary hormone group. The failure of our liver slice preparations to respond to purified crystalline growth hormone *in vitro* suggests,

in any case, that the results observed by Shipley *et al.* may not have been due to this hormone.

SUMMARY

The effects of various hormonal manipulations upon the formation of ketone bodies by rat liver slices have been investigated. The following has been shown.

1. To obtain true rates of production, allowance must be made for the preformed ketone bodies in the liver slice.
2. Removal of the adrenal gland fails to depress the rate of formation of ketone bodies after 18 hours of fasting and may even raise it.
3. Removal of the hypophysis causes a reduction in the rate of formation of ketone bodies, which can be accounted for entirely on the basis of reduced activity of the thyroid gland. The effects of hypophysectomy can be returned to normal by the administration of thyroxine.
4. Adrenalectomy prevents the normal rise of blood ketone body concentration from the 18th to the 40th hour of fasting. During this time there is also a significant fall in the rate of production of ketone bodies by liver slices.
5. The administration of crystalline anterior pituitary growth hormone *in vitro* or *in vivo* and of adrenocorticotrophic hormone *in vivo* does not alter the Q_{acac} .

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THE LOSS AND REGENERATION OF RAT LIVER ENZYMES RELATED TO DIET PROTEIN*

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This report extends previous findings demonstrating a relation between the loss of liver enzyme activity and the loss of liver protein resulting from complete inanition (1).

The data presented here support the view that specific liver enzyme proteins are lost during maintenance on diets causing a loss of liver protein and that a high protein diet, favoring the restoration of liver protein in general, favors an equally rapid regeneration of liver enzymes (*i.e.*, activity). In short, cellular enzymes participate as protein in the dynamic exchanges of protein metabolism (2, 3).

In addition to catalase, alkaline phosphatase, cathepsin, and xanthine dehydrogenase, liver arginase has been studied under conditions allowing a reexamination of the notion that changes in liver arginase activity are an adaptive response to changing needs for urea-forming capacity (4). This was done by comparing the livers of rats maintained on an adequate diet (25 per cent protein) with those of animals maintained on a diet in which the major portion of the protein was replaced with the amino acid glycine. This rendered the diet unsatisfactory for growth without greatly altering the N content and is to be compared with Lightbody and Kleinman's diet containing 18 per cent gelatin and 6 per cent casein (4). It will be seen that this lack of diet protein, biologically sufficient to maintain liver protein, is associated with decreased liver arginase activity in spite of a high dietary amino N content.

Methods

Male and female rats of Sherman strain, weighing 225 to 350 gm. and 175 to 250 gm. respectively, were maintained on a stock synthetic diet for at least 10 days prior to their use. The diet contained vitamin-free casein (General Biochemicals, Inc.) 25 per cent, glucose, Merck's anhydrous, 61 per cent, Mazola oil 10 per cent, salt mixture U. S. P. XII (General Biochemicals, Inc.) 4 per cent, and the following vitamins¹ per kilo:

* Part of this report was presented at the meeting of the American Society of Biological Chemists at Atlantic City, March 17, 1948 (*Federation Proc.*, 7, 174 (1948)).

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¹ We are grateful to Merck and Company, Inc., Rahway, New Jersey, for a generous supply of vitamins used in these experiments.

thiamine 10 mg., riboflavin 10 mg., pyridoxine 10 mg., calcium pantothenate 30 mg., niacin 25 mg., choline chloride 1 gm., menadione 4 mg., inositol 500 mg., vitamin A 60,000 i.u., and vitamin D 1500 i.u.

The *low protein diet* was prepared to contain 6 per cent casein and 80 per cent glucose and was in other respects identical with the above control diet. The rats were maintained on this *low protein diet* for 21 or 23 days before sacrifice by stunning and exsanguination.

The *non-protein diet* contained no protein whatsoever, 86 per cent glucose, and was in other respects identical with the control diet. The effect of the *non-protein diet* was observed in the rats sacrificed after 14 or 16 days.

The "refed" rats were first fed the low protein diet for 23 days, transferred to the non-protein diet for 9 days, and then refed the 25 per cent casein basal diet for 3 days (four rats) or 7 days (four rats). Since the enzyme activities and protein content of the livers of both groups were not significantly different, the results are presented as a single group in Tables I to VI.

Only female rats were used in the low protein, non-protein, and refeeding experiments and controls.

Only male rats were used in the experiment on the changes in arginase (and the other enzymes) in Table VI. Here the 25 per cent casein basal diet was replaced by one containing 6 per cent casein, 19 per cent glycine (c.p., Merck), and the other constituents in amounts identical with those of the other diets. The rats on this high glycine diet were sacrificed after 21 or 23 days. It was noted that the rats in this group became unkempt, developed alopecia with a "hooded" distribution, and swelling of the feet. These changes are suggestive of those ascribed to a relative pyridoxine deficiency in rats receiving excessive amounts of dietary glycine (5).

At least one control rat was sacrificed and its liver analyzed along with livers from each of the several experimental groups in each day's work.

The methods for preparing the liver homogenate and for the estimation of liver catalase, alkaline phosphatase, cathepsin, xanthine dehydrogenase, and liver protein were identical with those previously used (1). Arginase activity was estimated by the method of Folley and Greenbaum (6) without the addition of metal activators. The urea released was determined after urease treatment by the method of Conway (7). Units of activity are equivalent to mg. of urea liberated per hour per gm. of liver, per gm. of liver protein, or per 100 gm. of initial body weight, as the case may be.

Results

Table I presents the data on the liver catalase activity and makes clear that liver catalase activity is lost while the rats are maintained on the low

protein diet, and to an even greater extent on the non-protein diet. The loss of activity is most marked when expressed on the basis of initial body weight. The catalase (activity) declines somewhat more rapidly than the liver protein in general. Such a comparison is permissible if one may

TABLE I
*Liver Catalase Activity**

Diet	No. of rats	Units per gm. liver	Units per 100 gm. initial body weight	Units per gm. liver protein	Calculated,† units per gm. liver protein	Mg. liver protein per 100 gm. initial body weight
Control	5	2040 ± 710	7490 ± 2570	9,940 ± 3520		745
Low protein.	6	1660 ± 350	5730 ± 1210	9,770 ± 2090	12,900	573
Non-protein.	9	1250 ± 450	3110 ± 1110	7,330 ± 2440	17,000	437
Refed.	8	2130 ± 1100	8050 ± 3060	11,200 ± 5100		755

* The data in this and subsequent tables are given with the calculated standard deviation from the mean from the equation standard deviation = $\pm \sqrt{\Sigma(x - \bar{x})^2 / (n - 1)}$, where n is equal to the number of rats in a group.

† Calculation of expected mean activity on the assumptions that none of the initial enzyme activity is lost and that enzyme protein is an insignificantly small part of total protein

TABLE II
*Liver Alkaline Phosphatase Activity**

Diet	No. of rats	Units per gm. liver	Units per 100 gm. initial body weight	Units per gm. liver protein	Calculated, units per gm. liver protein	Mg. liver protein per 100 gm. initial body weight
Control.	5	0.86 ± 0.08	3.10 ± 0.25	4.16 ± 0.31		745
Low protein	5	0.81 ± 0.11	2.81 ± 0.37	4.79 ± 0.26	5.41	573
Non-protein	9	1.05 ± 0.16	2.60 ± 0.28	6.16 ± 1.20	7.12	437
Refed.	8	1.02 ± 0.18	3.92 ± 0.70	5.33 ± 0.84		755

* See foot-notes to Table I.

assume that the total catalase enzyme protein is insignificantly small in comparison with the total liver protein.

Refeeding of the 25 per cent casein diet resulted in the restoration of the enzyme and liver protein in general to levels indistinguishable from those of the normal controls.

Table II reveals changes in liver alkaline phosphatase activity incident to maintenance on the low protein and non-protein diets. It appears that

on a basis of units per gm. of liver protein there is some preferential conservation of the phosphatase activity, but the activity calculable on the basis of no loss of the enzyme protein indicates that such preferential conservation is not complete in the face of general liver protein loss. Refeeding a diet adequate in protein restores the alkaline phosphatase activity to levels somewhat above those noted for the controls. The quantitative significance of this change in excess of normal levels with refeeding is questionable, but the restoration of activity is clearly prompt and definite.

As a result of maintenance on the low protein diet for 9 days, the rats show changes in liver xanthine dehydrogenase activity as extreme as those previously found to result from 7 days complete inanition (1) (see Table III). Because the xanthine dehydrogenase activity of the livers in this

TABLE III
*Liver Xanthine Dehydrogenase Activity**

Diet	No. of rats	Units per gm. liver	Units per 100 gm. initial body weight	Units per gm. liver protein	Calculated, units per gm. liver protein	Mg liver protein per 100 gm. initial body weight
Control†. . . .	10	2.0	7.0 ± 2.4	10.2 ± 3.3	10.8	668
Low protein‡. . .	6	0.33	1.2	1.9		632
Refed	8	1.5 ± 0.5	5.7 ± 2.2	7.8 ± 2.9		755

* See foot-notes to Table I.

† For comparison, control data are taken from Table III of Miller (1).

‡ The rats used for the xanthine dehydrogenase *low protein* study were sacrificed after only 9 days on the diet.

group was so low, no attempt was made to study the livers of the rats fed the non-protein diet. In spite of the disproportionately great loss of xanthine dehydrogenase activity, rapid restoration of activity to a level of about 75 per cent of that initially present occurs within the short refeeding period.

Based on the activity in terms of units per gm. of liver protein, consuming the 6 per cent casein diet is associated with insignificant loss of cathepsin activity (Table IV) since the observed activity is not significantly different from the activity calculated on the basis that the cathepsin activity is preferentially retained, while the liver protein in general is lost. Maintenance of the rats on the non-protein diet resulted in a marked loss of cathepsin activity, whether the comparison with the control values is made either on the basis of activity per 100 gm. of initial body weight or on the basis of activity per gm. of liver protein. As noted above with the

other enzymes studied, refeeding results in prompt restoration of the cathepsin activity to initial control values.

Table V shows that low protein or non-protein diets affect the liver arginase activity to a degree comparable with liver catalase. The arginase is not preferentially spared even on the low protein diet, and the activity calculated (assuming no enzyme is lost with the liver protein) is considerably in excess of the observed activity. The disparity is even more

TABLE IV
*Liver Cathepsin Activity**

Diet	No. of rats	Units per gm. liver	Units per 100 gm. initial body weight	Units per gm. liver protein	Calculated, units per gm. liver protein	Mg. liver protein per 100 gm. initial body weight
Control . . .	5	26.7 \pm 7.9	98.6 \pm 37	130 \pm 41		745
Low protein	5	27.4 \pm 3.8	94.9 \pm 12.4	163 \pm 18	169	573
Non-protein	9	23.7 \pm 7.9	57.5 \pm 16.9	136 \pm 34.1	222	437
Refed.	8	23.9 \pm 4.4	92.4 \pm 19.3	124 \pm 19.1		755

* See foot-notes to Table I.

TABLE V
*Liver Arginase Activity**

Diet	No. of rats	Units per gm. liver	Units per 100 gm. initial body weight	Units per gm. liver protein	Calculated, units per gm. liver protein	Mg. liver protein per 100 gm. initial body weight
Control	5	148 \pm 19	536 \pm 45	722 \pm 77		745
Low protein	6	127 \pm 18	433 \pm 83	750 \pm 124	938	573
Non-protein	9	122 \pm 18	309 \pm 64	683 \pm 127	1230	437
Refed.	8	127 \pm 13	444 \pm 81	663 \pm 71		755

* See foot-notes to Table I.

marked on the non-protein diet. Refeeding results in obviously extensive regeneration which appears to lag somewhat behind the liver protein regeneration.

Table VI presents data bearing on the question of enzyme adaptation in higher mammals. A consideration of the data on changes in liver arginase activity after 23 days on the 19 per cent glycine and 6 per cent casein diet shows an *absolute* decrease in arginase activity comparable with the loss of activity of the other enzymes from the same livers. One excep-

tion, *viz.* that of alkaline phosphatase, is apparent; the phosphatase activity is maintained or slightly increased. The interpretation of changes in

TABLE VI
Lack of Enzyme Adaptation

Diet	No. of rats	Units per gm. liver	Units per 100 gm. initial body weight	Units per gm. liver protein	Calculated,* units per gm. liver protein
Liver arginase activity					
Control	4	161 \pm 14	523 \pm 45	779 \pm 58	1000
Casein 6 per cent + glycine 19 per cent	8	128 \pm 20	329 \pm 63	621 \pm 82	
Liver catalase activity					
Control	4	2870 \pm 550	9350 \pm 1820	14,700 \pm 3400	18,900
Casein 6 per cent + glycine 19 per cent	8	2390 \pm 1000	6000 \pm 2100	11,620 \pm 3200	
Liver alkaline phosphatase activity					
Control	4	1.47 \pm 0.14	4.78 \pm 1.3	7.13 \pm 2.3	9.18
Casein 6 per cent + glycine 19 per cent	8	2.01 \pm 0.20	5.19 \pm 0.63	9.99 \pm 1.0	
Liver xanthine dehydrogenase activity					
Control	4	2.7 \pm 0.8	8.9 \pm 2.6	13.7 \pm 4.5	17.6
Casein 6 per cent + glycine 19 per cent	8	0.46 \pm 0.2	1.1 \pm 0.4	2.3 \pm 0.1	
Liver cathepsin activity					
Control	4	21.8 \pm 5.4	70.2 \pm 14.2	106 \pm 26	135
Casein 6 per cent + glycine 19 per cent	8	17.1 \pm 2.4	45.0 \pm 3.6	87.4 \pm 7.2	

* Calculation of expected mean activity on the assumptions that none of the initial enzyme activity is lost and that enzyme protein is an insignificantly small part of total protein. Calculation based on mean liver protein of 665 and 517 mg. per 100 gm. of initial body weight in rats on control and glycine diets respectively.

the phosphatase activity in these experiments is subject to at least two complicating factors. First, the contribution of biliary tract epithelium to the alkaline phosphatase of a liver homogenate is a real but, as yet,

unevaluated factor. Secondly, glycine, known to have an effect on bone alkaline phosphatase described by Bodansky (8), may have a similar action on liver alkaline phosphatase which we did not attempt to control.

It appears that the loss of liver arginase activity is consistent with the general loss of enzyme activity noted previously under dietary conditions leading to a loss of liver proteins in general and enzyme proteins in particular.

DISCUSSION

The validity of the assumption that changes in enzyme activity in these experiments represent changes in the amount of enzyme protein *per se* has been discussed (1), and the arguments supporting the assumption will not be repeated here. Nor will we dwell on the merits of the several ways of expressing the enzyme activities used in Tables I to VI.

It is worth emphasizing that, if the five hepatic enzymes studied, chosen at random, decreased under dietary conditions leading to liver protein loss, many other, if not all, liver enzymes decrease similarly in activity under adverse dietary conditions. This may be regarded as a reduction in the functional reserve of the organism and may become of critical significance when the organism is subjected to abnormal stresses imposed by toxic or disease agents.

Since these observations were first reported (9), other workers have noted analogous results in studies of rabbit liver and kidney cathepsin (10), succinic dehydrogenase and octanoate oxidase (11), rat liver rhodanese, adenosinetriphosphatase and arginase (12), and D-amino acid oxidase (13).

The observations above point to the conclusion that protein refeeding leads to the restoration of liver protein and this is followed or accompanied by a simultaneous restoration of liver enzyme protein (*i.e.*, activity).

The apparently rapid restoration of the liver enzymes to or toward normal levels with refeeding a diet containing adequate protein deserves comparison with the findings of Kaplansky *et al.* (14). The latter observed that if refeeding an adequate diet were delayed too long (beyond 25 days) some of the rats failed to regain normal glycogen-forming or deaminating functions; in fact, many of the animals became progressively worse and succumbed. This implies that protein deprivation eventually may lead to irreversible damage of the protein- (*i.e.*, enzyme-) synthesizing mechanisms, whatever they may be.

The above observations on liver enzyme loss in rats fed diets containing protein qualitatively or quantitatively inadequate for growth or maintenance of body protein stores afford a rational basis for understanding the impaired capacity of such animals to withstand hepatotoxic agents (15), as well as of normally well fed animals, and the restoration of the liver toward normal with the restoration of adequate protein nutrition.

The occurrence of enzyme adaptation is well established as a phenomenon conditioning the survival and propagation of microbiological species. Equally convincing evidence for the occurrence of enzyme adaptation in higher mammals is lacking. On the basis of the above experiments it appears that the liver enzyme arginase cannot be maintained at normal levels in the absence of biologically adequate diet protein; the fact that the liver arginase decreases in the presence of a continued high dietary N content does not support Lightbody and Kleinman's (4) conclusion that rat liver arginase responds adaptively to varying dietary N levels. Rather, it affirms the notion that cellular enzyme proteins like the body and plasma proteins are normally in a state of dynamic exchange with the metabolites involved in protein synthesis and breakdown. Further, factors favoring protein synthesis will promote enzyme synthesis, and factors favoring a progressive loss of body protein will result in the loss of cellular enzyme proteins in greater or lesser measure.

SUMMARY

1. Maintenance of rats on low or non-protein diets is associated with the loss of liver protein and liver catalase, alkaline phosphatase, xanthine dehydrogenase, cathepsin, and arginase activities. Refeeding a high protein diet leads to prompt restoration of both liver protein and enzyme activities.

2. These results support the notion that liver enzymes, as proteins, participate in a dynamic non-specific manner in the metabolism of proteins in general.

We acknowledge the able assistance of Miss Bernice Deitch in the above experiments.

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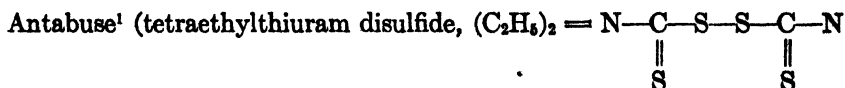
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THE COMPOSITION OF RAT LIVER XANTHINE OXIDASE AND ITS INHIBITION BY ANTABUSE*

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was recently introduced as a treatment for chronic alcoholism by a group of Danish investigators (1, 2). By itself it is without any prominent pharmacological action, but subsequent consumption of alcohol produces nausea, vomiting, and a variety of symptoms and effects (1-5). These symptoms are accompanied by an elevated blood acetaldehyde (6) and can be reproduced by acetaldehyde administration (7). The fundamental action of the drug appears to be an inhibition of the metabolism of the acetaldehyde that is produced metabolically from the alcohol.

There are five different enzyme systems within the body that are capable of utilizing acetaldehyde as a substrate (aldolase, mutase, carboxylase, aldehyde oxidase, and xanthine oxidase). Since the liver is the most important organ concerned with acetaldehyde metabolism *in vivo* (8) and is generally a good source of mutase, aldehyde oxidase, and xanthine oxidase, one or more of these three enzymes would seem likely to be of major importance in acetaldehyde metabolism. It is possible to produce a defect in acetaldehyde metabolism in dogs by dietary means (9), and a similar dependence of rat liver xanthine oxidase upon an adequate diet has been demonstrated (10).

This report describes the inhibition of xanthine oxidase in rat liver by antabuse; milk xanthine oxidase was not affected. The evidence further indicated that the xanthine oxidase occurred in rat liver as a complex of two or more chemical entities and two independent enzymatic activities: (1) a dehydrogenase action capable of transferring hydrogen from xanthine to methylene blue, and (2) an oxidase action that was responsible

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¹ The antabuse was supplied by Ayerst, McKenna and Harrison, Ltd.

for reoxidation of the reduced enzyme by atmospheric oxygen. Only the oxidase portion of the enzyme was inhibited by antabuse, apparently because that portion of the enzyme was bound to some antabuse-sensitive constituent that could be removed or inactivated by heating at 56°.

EXPERIMENTAL

Methods—The activity of liver xanthine oxidase was determined by the method of Axelrod and Elvehjem (11) as previously described (12). The enzyme activity in whole raw milk and in various preparations of purified xanthine oxidase from milk (13) and liver was tested similarly. Antabuse was homogenized with a portion of the liver homogenate, whole milk, or the phosphate buffer added to the purified enzyme. The final concentrations of antabuse in mg. per cc. of fluid are given with the results; the Warburg flask contained 283 mg. of fresh liver in a total of 2.0 cc. of fluid. In those experiments containing methylene blue, 0.15 cc. of 0.0113 M methylene blue was part of the 2.0 cc. fluid volume. When heated at 56°, the liver was homogenized with 2.5 volumes of buffer, instead of the usual 5 volumes, and placed in a 56° water bath for the indicated time. It was then rehomogenized with additional buffer or antabuse-containing buffer to give the same proportions used with the unheated liver.

Results

The addition of antabuse *in vitro* to a normal rat liver homogenate inhibited the xanthine oxidase activity in proportion to the amount of antabuse added and the original activity of the liver. In a liver preparation whose net O₂ uptake with xanthine in the absence of antabuse was 44.5 c.mm. of O₂ per 20 minutes, the addition of antabuse reduced this activity as follows: 0.1 mg. of antabuse per cc. gave an activity of 18 c.mm. of O₂ per 20 minutes; 0.4 mg. per cc. gave 15 c.mm.; 0.85 mg. per cc. gave 8 c.mm.; and 2.5 mg. per cc. gave 4.5 c.mm. In the same homogenate 0.85 mg. of antabuse per cc. had no effect on the oxidation of urate; hence uricase was unaffected by antabuse and was not secondarily responsible for any portion of the altered xanthine oxidase activity. The effect of 0.2 and 0.4 mg. of antabuse per cc. was studied more extensively in a group of normal rat livers and the liver xanthine oxidase was found to be inhibited, on the average, 58 and 71 per cent respectively (Experiment 1, Table I). At a fixed concentration of antabuse, the percentage inhibition of the liver xanthine oxidase was greater when the original enzyme activity was low. This greater inhibition of smaller amounts of enzyme may have been a mass action effect, or it may have been due to the fact that livers of low xanthine oxidase activity were inhibited to

the same degree as were the more active livers, but the small amount of activity remaining in the former could not be detected (12).

TABLE I
Effect of Antabuse Added in Vitro on Xanthine Oxidase

Experiment No.	Preparation tested	Antabuse concentration	Xanthine oxidase activity	Per cent inhibition	Ratio, XOA* recovered milk XOA added
		mg. per cc.	c.mm. O ₂ per 20 min.		
1	Normal rat liver, † 1680 ± 94 units	0	47.8 ± 2.68		
		0.20	20.2 ± 2.56	58	
		0.40	13.8 ± 3.84	71	
2	Whole raw milk	0	41		
		5.0	38	7	
		2.5	41	0	
		0.25	41	0	
		0	35		
3	Purified milk xanthine oxidase	1.0	36	0	
		0	51		
4	Normal rat liver, 1790 units	0.85	24	53	
		0	26		
		0	>80		>1.1
5	Rat liver, ‡ 950 units	0.85	65		1.6
		0	27		
		0.85	0	100	
		0	47		
		1.0	45	4	
6	Liver + milk xanthine oxidase	0	82		1.2
		0.85	60		1.3
		0	0		
		0.85	0		
		0	32		
6	Rat liver, ‡ 0 units	1.0	31	3	
		0	46		1.4
		0.85	37		1.2
		0	32		
		1.0	31		

* XOA, xanthine oxidase activity.

† Averages for four male and four female rats maintained on dog chow and weighing an average of 326 gm.

‡ Liver from rat maintained for 2 weeks after weaning on a purified 21 per cent casein diet (Experiment 5) or 8 per cent casein diet (Experiment 6).

Milk xanthine oxidase was not inhibited by antabuse. Various preparations such as whole milk, the 60 per cent and the 33 to 42 per cent saturated ammonium sulfate precipitates of milk xanthine oxidase prepared by Ball's procedure (13) were not inhibited at all by suspensions

of antabuse in a concentration of 0.25 to 5.0 mg. per cc. (Experiments 2 and 3, Table I).

This difference between the effect of antabuse on the xanthine oxidases from milk and liver indicated that either the two enzymes were different, or the antabuse itself was not the enzyme inhibitor but was converted to an inhibitor by the liver homogenate. To test these possibilities, the purified milk xanthine oxidase was added to three different rat liver homogenates of differing xanthine oxidase activities; antabuse was also added *in vitro*. The results (Experiments 4, 5, and 6, Table I) showed that the antabuse inhibited the liver enzyme in the manner described above, but the activity of the added milk enzyme was unimpaired in the presence of the same liver homogenate. The increased activity of the milk enzyme when added to the liver homogenate was also noted in the presence of antabuse. The factors responsible for this increased activity, possibly uricase and catalase (11), were not inhibited by antabuse. Hence, no evidence was obtained for the conversion of antabuse to an inhibitor equally effective against both enzymes.

The effect of adding antabuse *in vitro* to a normal rat liver homogenate is shown in Fig. 1 (averages from Experiment 1, Table I). *In vitro*, antabuse had a profound effect in inhibiting the endogenous respiration of the liver, which was much more than could be accounted for by the inhibition of xanthine oxidase alone. With increasing concentrations of antabuse added *in vitro* (0.4 to 2.5 mg. per cc.), the inhibition of the endogenous respiration became progressively greater. The tendency for this endogenous respiration to escape from the antabuse inhibition in time is evident in Fig. 1 and was still more striking when larger amounts of antabuse were added, because the early inhibition was then more profound. Similarly, the activity of the antabuse-inhibited liver homogenate toward xanthine may have been negligible for an hour after the latter was tipped in before some activity became apparent. When this happened, the activity usually became stabilized at about 50 per cent of the activity found in the absence of antabuse.

Effect of Methylene Blue—Methylene blue increased the aerobic xanthine oxidase activity of a homogenate because the liver xanthine oxidase was able to remove hydrogen from the substrate faster than the reduced enzyme could be reoxidized by air (12). When added to a liver homogenate containing antabuse, methylene blue overcame the antabuse inhibition of xanthine oxidase almost completely. A typical experiment is shown in Fig. 2. The effect of methylene blue on the endogenous oxygen consumption curve, previously described (12), is noted, and the marked early effect of antabuse on this curve is shown. The presence of antabuse and methylene blue together showed little effect of the methylene

blue on the inhibition of the endogenous oxygen consumption by antabuse, but almost a complete restoration of the xanthine oxidase activity of the liver homogenate.

This experiment showed that the enzyme was capable of removing hydrogen from xanthine in the presence of antabuse, but passing the hydrogen on to oxygen was inhibited; hence a decreased aerobic activity resulted. When methylene blue was also present to accept the hydrogen from the reduced xanthine oxidase and to pass it on to oxygen, a relatively

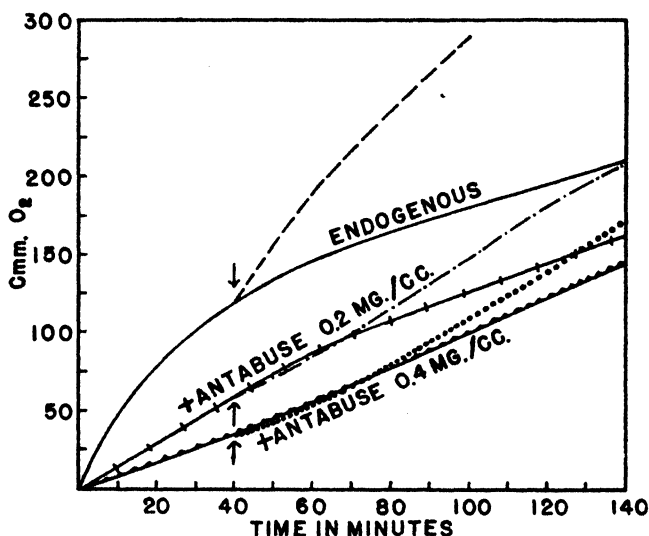


FIG. 1. Average oxygen consumption curves of rat liver homogenates after addition of antabuse *in vitro*. Solid line, endogenous respiration of normal rat liver homogenate; xanthine tipped in at arrow to give the broken line. The other curves show the effect of adding 0.2 and 0.4 mg. of antabuse per cc. of medium. Xanthine oxidase activities, control, 47.8 c.mm. of O_2 per 20 minutes (1680 units); 0.2 mg. of antabuse per cc., 20.2 c.mm. of O_2 per 20 minutes (710 units); 0.4 mg. of antabuse per cc., 13.8 c.mm. of O_2 per 20 minutes (485 units).

normal activity was found. The antabuse inhibited only that portion of the enzyme that was concerned with its reoxidation.

Effect of Heating—The antabuse-sensitive portion of the liver xanthine oxidase complex was disrupted by short term heating of the homogenate at 56° , since the only effect observed was a decrease or disappearance of the antabuse effect. Longer heating also gradually reduced the capacity of the enzyme to oxidize xanthine aerobically, but had no effect on its dehydrogenase activity in the presence of methylene blue. Individual livers varied appreciably in the extent to which a 5 minute heating of the homogenate eliminated the antabuse inhibition, and in the extent to

which the aerobic oxidation of xanthine was affected by either a 5 or 30 minute heating. All livers responded similarly in giving the same xanthine oxidase activity in the presence of methylene blue after heating as was observed prior to heating, and in the consistent elimination of the antabuse effect with longer heating.

The average results obtained in six experiments, Table II, show the gradual loss of aerobic xanthine oxidase activity and a disappearance of

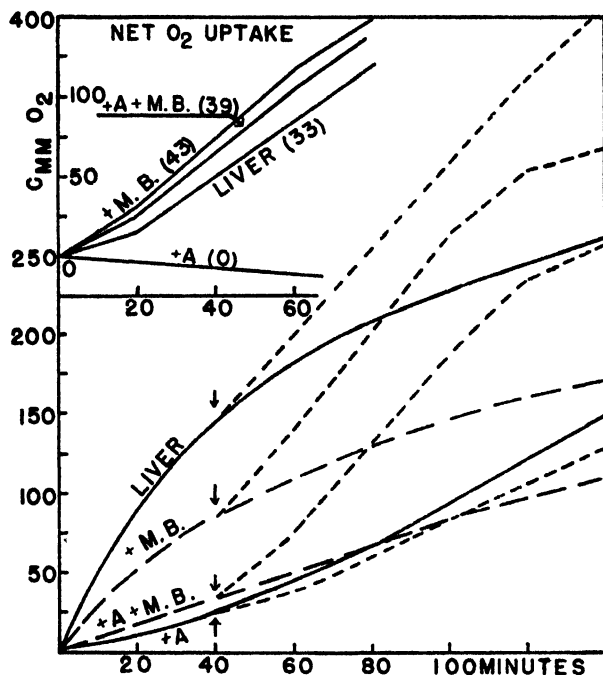


FIG. 2. The effect of methylene blue on the inhibition of liver xanthine oxidase by antabuse, showing the endogenous oxygen consumption of a liver homogenate alone (liver), with 0.15 cc. of 0.0113 *M* methylene blue (*M.B.*), 0.85 mg. of antabuse per cc. (*A*), and both together. Xanthine was tipped in at the arrows to give the dotted lines. The net oxygen consumption due to xanthine is shown in upper left-hand corner. The figures in parentheses refer to c.mm. of O₂ per 20 minutes.

the antabuse inhibition with heating. The original xanthine oxidase activity in the presence of methylene blue was unaffected by the antabuse or the heating (in one experiment after 2 hours at 56°). Heating the average liver homogenate at 56° for 5 minutes only partially destroyed the antabuse sensitivity, but did not otherwise affect the xanthine oxidase. Heating for 10 minutes or longer completely eliminated the antabuse effect, but also partially inactivated the aerobic activity of the enzyme.

The oxygen consumption curves for an unheated liver homogenate and a portion heated at 56° for 15 minutes are shown in Figs. 2 and 3. The curves after heating probably result from the partial destruction of some enzymes and the exhaustion of some of the endogenous substrates during the heating period.

Purified Liver Xanthine Oxidase—As a further test of the validity of the experiments on crude homogenates, xanthine oxidase was extracted from rat livers, partially purified, and the previous experiments repeated. The following method of preparation was used. Pooled livers from normal rats were homogenized and extracted with 2.5 volumes of 0.2 M Na_2HPO_4 . Saturated ammonium sulfate, adjusted to pH 7.35 with ammonia, was added to give a 30 per cent saturated solution. By prolonged centrifugation in the cold and repeated extraction of the incompletely packed pre-

TABLE II
Effect of Methylene Blue and Antabuse on Xanthine Oxidase Activity of Liver Homogenates Heated at 56°

	Xanthine oxidase activity, c.mm. O ₂ per 20 min.			
	Un- heated	Heated at 56° for		
		5 min.	10 min.	30 min.
Liver.....	34	32	20	15
“ + methylene blue.....	60	66	62	55
“ + antabuse (0.85 mg. per cc.).....	12	24	19	19
“ + “ + methylene blue.....	60	62	62	62

cipitate with 30 per cent saturated $(\text{NH}_4)_2\text{SO}_4$, about 80 per cent of the original activity was obtained in the decanted supernatant. The supernatant was then made 60 per cent saturated with ammoniacal $(\text{NH}_4)_2\text{SO}_4$, and centrifuged in the cold. The precipitate, containing about half of the original activity, was dissolved in water and reprecipitated at 42 to 60 per cent saturated ammoniacal $(\text{NH}_4)_2\text{SO}_4$. This precipitate was dialyzed for approximately 18 hours in redistilled water in the cold, and was then centrifuged. The supernatant contained about one-fourth of the original activity, and was again precipitated between the limits of 42 to 60 per cent saturated ammoniacal $(\text{NH}_4)_2\text{SO}_4$. The precipitate was again dialyzed and centrifuged; the supernatant solution contained about 17 per cent of the original activity and represented the highest purification attempted.

The effect of antabuse and methylene blue on the activity of the liver xanthine oxidase was tested at each step in the purification; in many

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cases the small amount of activity in the discarded fractions was also tested in the presence of methylene blue and antabuse to see whether any evidence could be obtained for the existence in liver of more than one component of this enzyme complex. No such evidence was obtained, and the enzyme responded to the fractionation procedure as a single unit. Both the antabuse effect in inhibiting the liver enzyme and the methylene

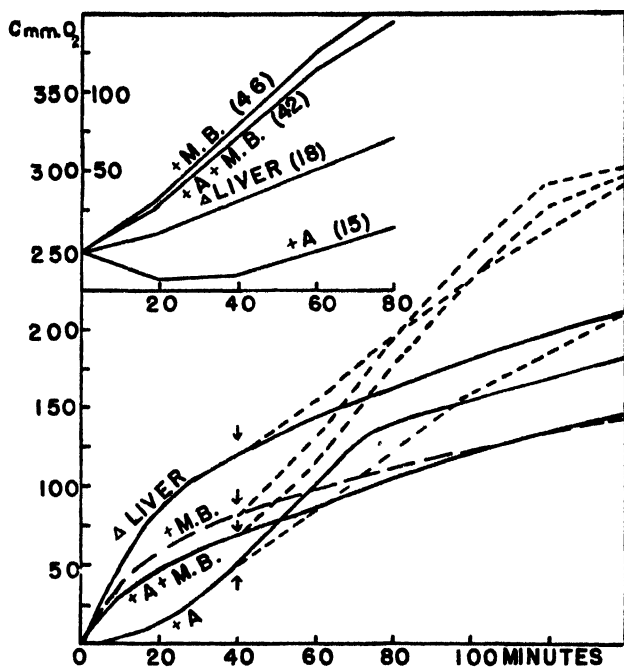


FIG. 3. The effect of heating at 56° on the antabuse inhibition of rat liver xanthine oxidase. A portion of the same rat liver shown in Fig. 2 was homogenized with 2.5 volumes of buffer and heated in a 56° bath for 15 minutes. Aliquots were then diluted with buffer alone or with buffer plus antabuse to give the same concentrations used in Fig. 2. Xanthine was tipped in at the arrows to give the dotted lines. The net oxygen consumption due to xanthine is shown in upper left-hand corner. The figures in parentheses refer to c.mm. of O₂ per 20 minutes. M.B., methylene blue; A, antabuse.

blue effect in increasing the enzyme activity remained constant during the purification. The xanthine oxidase activity of the original liver homogenate and of each of the purified fractions was inhibited approximately 50 per cent (41 to 58 per cent) by an antabuse concentration of 0.85 mg per cc. of medium. Methylene blue increased the xanthine oxidase activity of the original homogenate and of each of the purified fractions to approximately the same degree, 1.6-fold. No endogenous oxygen con-

sumption was exhibited by the purified enzyme preparations in the presence or absence of antabuse. The results obtained with the original liver homogenate and with the most highly purified fraction are given in Table III.

The most highly purified liver fraction was heated at 56° for 30 minutes, and the insoluble precipitate was removed by centrifugation. Testing the supernatant with antabuse and methylene blue, Table III, showed that the antabuse effect was eliminated, but the enzyme was otherwise unaffected. In contrast to the homogenate, the prolonged heating of the purified enzyme did not decrease its autoxidizable characteristics.

Heating a purified xanthine oxidase from milk at 56° not only reduced the aerobic activity of the enzyme but also, decreased its activity in the

TABLE III

Effect of Antabuse and Methylene Blue on Purified Rat Liver Xanthine Oxidase

	Xanthine oxidase activity, c.mm. O ₂ per 20 min.		Per cent inhibition
	No antabuse	0.85 mg. per cc. antabuse	
Original liver homogenate, 1190 units.....	34	20	41
" " + methylene blue.....	58	55	5
Purified " xanthine oxidase.....	29	12	59
" " " " + methylene blue.. . .	42	43	0
Δ purified liver xanthine oxidase*.....	28	29	0
" " " " " + methylene blue. . .	50	49	2

* Sample in 0.04 M phosphate buffer, pH 7.4, heated at 56°; insoluble precipitate removed by centrifugation and supernatant tested.

presence of methylene blue proportionally. After heating a preparation (whose original activity in the presence and absence of methylene blue was 42:20 c.mm. of O₂ per 10 minutes) for 10, 15, 25, and 45 minutes, the corresponding values were 30:16, 25:11.5, 20:9.5, and 12:7.

Oxidation of p-Hydroxybenzaldehyde—Milk xanthine oxidase dehydrogenates *p*-hydroxybenzaldehyde as readily as hypoxanthine in the methylene blue procedure (14). The oxidation of this aldehyde by rat liver homogenates was studied by substituting 0.15 cc. of a 0.05 M solution of *p*-hydroxybenzaldehyde for the xanthine in an otherwise identical system. The net oxygen consumption following the tipping in of the aldehyde was maximal during the first 20 minutes but decreased rapidly thereafter; others have noted the rapid destruction of the enzyme by aldehydes. The net O₂ consumption with the aldehyde substrate during the first

20 minutes was 68 per cent of the linear rate obtained with xanthine (average of thirty-four rat livers tested simultaneously with *p*-hydroxybenzaldehyde and xanthine). A comparison of the inhibition produced by various concentrations of antabuse on the oxidation of the two substrates is shown in Table IV. The oxidation of the aldehyde by the liver homogenate was more effectively inhibited by the antabuse than was the oxidation of xanthine, but the minimal effective concentration was about the same for both substrates.

TABLE IV
Effect of Antabuse on Oxidation of p-Hydroxybenzaldehyde and Xanthine by Rat Liver Homogenates

	Per cent inhibition of activity				
	Antabuse, mg. per cc.				
	0.5	0.25	0.05	0.025	0.0125
Xanthine.....	85	56	35	16	2
<i>p</i> -Hydroxybenzaldehyde.....	100	100	76	41	2

TABLE V
Comparative Effect of Methylene Blue and Antabuse on Oxidation of Xanthine and p-Hydroxybenzaldehyde by Rat Liver Homogenates

	Net oxygen uptake, c.mm. O ₂ per 20 min.			
	Xanthine substrate		<i>p</i> -Hydroxybenzaldehyde substrate	
	No antabuse	Antabuse, 0.85 mg. per cc.	No antabuse	Antabuse, 0.85 mg. per cc.
Liver.....	28	2	21	0
" + methylene blue.....	57	52	67	41

The comparative effect of methylene blue and antabuse additions on the oxidation of xanthine and *p*-hydroxybenzaldehyde is shown in Table V (average of six experiments). Methylene blue increased the rate of oxidation of the aldehyde over 3-fold, but gave only a 2-fold increase in the presence of antabuse.

Additional Studies—The xanthine oxidase in homogenates of normal spleen and lung was also inhibited by antabuse. A concentration of 0.85 mg. per cc. decreased the net oxygen consumption with xanthine from 14 c.mm. of O₂ per 20 minutes to 7 for spleen, and from 9 to 3 for lung (average of three experiments). With both tissues there was a nearly

complete inhibition of the endogenous respiration for the first 40 minutes, after which there was a slight but increasingly rapid oxygen uptake.

Measurement of the D-amino acid oxidase activity (15) of normal rat liver homogenates in the presence and absence of added antabuse showed that an antabuse concentration of 0.33 mg. per cc. (\approx 1 mg. per cc. of 1:6 liver homogenate) had little or no effect on this enzyme. Methylene blue did not increase the activity of the D-amino acid oxidase in the presence or absence of antabuse. Heating the homogenate for 15 minutes at 56° increased the D-amino acid oxidase activity by about 20 per cent, and the activity was again found to be uninhibited by antabuse.

Oxidation of succinate by a rat liver homogenate was completely inhibited by 0.25 mg. per cc. of antabuse, and the inhibition was not overcome by methylene blue, nor did methylene blue increase the succinoxidase activity of the original liver homogenate. Heating the liver homogenate for 15 minutes at 56° destroyed nearly all (90 per cent) of the succinoxidase activity; the small amount of activity remaining was still inhibited by antabuse.

Feeding antabuse to rats and determining the xanthine oxidase activity of the liver gave variable results. In most experiments no significant effect was obtained. In several experiments the feeding of 100 to 350 mg. of antabuse per kilo of body weight per day for 1 to 7 days on a chow or purified diet reduced the liver xanthine oxidase activity to about one-third of the value found in pair-fed controls, the probability of chance variation being less than 1 in 100. Fed antabuse had no effect on the endogenous oxygen consumption of the liver homogenate unless the xanthine oxidase activity was also decreased; when the xanthine oxidase was inhibited, the endogenous oxygen consumption was depressed accordingly (12), but never to the extent observed in the experiments *in vitro*.

DISCUSSION

Liver and milk xanthine oxidases have been presumed to be similar enzymes. The only study of the purified liver xanthine oxidase (16) has supported this belief, but it should be noted that the purification procedure involved heating the preparation at 56°. The two enzymes are known to differ in solubility, the milk enzyme being precipitated by 30 to 42 per cent saturated ammonium sulfate, the liver enzyme by 42 to 60 per cent saturation. As a result of this study it is also known that the liver, but not the milk, xanthine oxidase is inhibited by antabuse. The evidence would seem to indicate that the liver enzyme may be similar to the milk enzyme, but that in its native state in liver it is attached to some other unidentified constituent. This attachment confers upon the complex a sensitivity to antabuse, for when the preparation is heated

briefly at 56° the xanthine oxidase activity is recovered essentially unchanged, and, like the milk enzyme, it is then no longer sensitive to antabuse. Presumably the mild heating frees the xanthine oxidase from the complex.

The evidence shows that the xanthine oxidase molecule is attached to the complex in such a way that the antabuse inhibition affects only the autoxidizable characteristic of the enzyme without affecting its dehydrogenase activity. Probably the attachment of the enzyme to the complex is through or near that portion of the xanthine oxidase molecule which is responsible for its reaction with oxygen, and a reaction between antabuse and the enzyme complex at this point in the structure blocks only this portion of the enzyme. A partial separation of dehydrogenase from oxidase activity of the xanthine oxidase can also be made in the homogenate by prolonged heating at 56°, whereby the capacity of the enzyme to react with air is gradually destroyed, but the dehydrogenase activity is retained. The presence of two different prosthetic groups in xanthine oxidase (13, 16) might be related to the separable dehydrogenase and oxidase functions of the enzyme.

The nature of the component of the liver xanthine oxidase complex that confers antabuse sensitivity to the complex has not been identified, but the possibility of its being phospholipide or ribonucleic acid is worthy of consideration. Succinoxidase appears to be bound to phospholipide (17) and ribonucleic acid (18), is completely inhibited by antabuse, and is destroyed by the mild heating procedure that frees xanthine oxidase from its complex. However, the addition to the Warburg flask of 12.5 or 25 mg. per cc. of the acetone-insoluble phospholipides extracted from rat liver with alcohol-ether did not prevent the antabuse inhibition of xanthine oxidase. Phospholipide actually increased the effectiveness of antabuse, possibly because of the better emulsification or solution of the inhibitor. Similarly the addition of 10 mg. of yeast nucleic acid per cc. did not prevent the inhibition of xanthine oxidase by antabuse.

There is no good evidence that xanthine oxidase is of major importance in acetaldehyde metabolism *in vivo*. This study lends some indirect support to the idea, but since the aldehyde oxidase is a very similar enzyme chemically, its inhibition by antabuse would not be unexpected. The possibility that still other enzymes are involved *in vivo* needs no emphasis.

SUMMARY

The addition of antabuse *in vitro* to a normal rat liver homogenate inhibited the xanthine oxidase activity in proportion to the amount of antabuse added and the original activity of the liver. 0.2 and 0.4 mg. of

antabuse per cc. gave an average 58 and 71 per cent inhibition, respectively. The endogenous respiration of the liver homogenate was markedly inhibited. Xanthine oxidase in normal rat spleen and lung was also inhibited.

Milk xanthine oxidase was not inhibited alone or when added to a liver homogenate containing antabuse. Methylene blue added to the liver homogenate in the aerobic test system overcame the inhibition of xanthine oxidase. This showed that the antabuse affected only that portion of the liver enzyme that was concerned with its autoxidation and did not inhibit its dehydrogenase activity.

Heating the liver homogenate at 56° for 5 minutes removed most of the antabuse sensitivity of the xanthine oxidase without otherwise affecting its activity, presumably because of the disruption of a xanthine oxidase complex in the liver. Heating for 30 minutes also gradually reduced the capacity of the enzyme to oxidize xanthine aerobically, but had no effect on its dehydrogenase activity in the presence of methylene blue.

Purified xanthine oxidase from rat liver responded like the homogenate in its inhibition by antabuse, its increased activity in the presence of methylene blue, the absence of antabuse inhibition in the presence of methylene blue, and the loss of antabuse sensitivity without otherwise affecting its activity after heating at 56°.

The oxidation of *p*-hydroxybenzaldehyde by a rat liver homogenate was more effectively inhibited by the antabuse than was the oxidation of xanthine. The D-amino acid oxidase activity of rat liver homogenates was not affected by antabuse, methylene blue, or heating at 56°. Succinoxidase activity was completely inhibited by antabuse; methylene blue had no effect on the original or antabuse-inhibited succinoxidase activity; heating the liver at 56° destroyed most of the succinoxidase activity.

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THE BIOLOGICAL FORMATION OF FORMATE FROM METHYL COMPOUNDS IN LIVER SLICES*

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Study of the biological synthesis of serine from glycine led us to infer that there is another source for the β -carbon of serine besides the α -carbon of glycine. The recent observations that serine is formed by a condensation of formate with glycine, both *in vivo* (1) and *in vitro* (2), and that the α -carbon of glycine gives rise to formate (2) led to the present investigation of another source of this formate. A first assumption would be that formate is produced by the oxidation or reduction of the 1-carbon entities of the body. Previously it had been found that carbon dioxide is not reduced to formate by rat liver slices (2). Work was undertaken, therefore, to determine whether the labile methyl groups of methionine or choline are oxidized to formate.

While the investigation was in progress, supporting evidence was published in a note by Sakami (3), who isolated serine containing C^{14} in the β -carbon from a rat injected with choline labeled in the methyl group.

Investigation of the reactions of *N*-methylamino acids by animal tissue preparations has yielded the following information: Handler, Bernheim, and Klein (4) obtained a colorimetric test for formaldehyde upon the oxidation of sarcosine to glycine and Mackenzie and du Vigneaud (5) observed the formation of radioactive formaldehyde from C^{14} -methyl-labeled sarcosine.

On the other hand, Keilin and Hartree (6) had previously found that the next higher homologue, *N*-methyl-DL-alanine, yields methylamine and pyruvic acid when acted upon by a preparation of D-amino acid oxidase. A similar reaction was found with the *N*-methyl derivatives of DL-methionine, DL-leucine, DL-alanine, and DL-histidine (7). L-Amino acid oxidase also catalyzes the oxidation of *N*-methylamino acids, *e.g.* of L-leucine,

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methionine, and homocysteine, to the corresponding keto acids and methylamine (8), while glycine oxidase catalyzes the oxidation of sarcosine to glyoxylic acid and methylamine (9). Recently, however, Ling and Tung (10) found that homogenates of kidney and liver of rabbit, guinea pig, and fowl oxidized many *N*-methyl-L-amino acids to the corresponding amino acids and formaldehyde, while the *D* isomers were not affected. This enzyme system, named demethylase, did not attack sarcosine or *N*-dimethyl-L-amino acids. Apparently the enzyme system which oxidizes glycine to formic acid (2) is not sarcosine oxidase, since the latter enzyme does not react further upon the glycine remaining (4). Recent experimental work has served to heighten the interest in formate metabolism. Knox and Mehler have found (11) that in the oxidation of L-tryptophan to kynurenine the carbon which is split off is released as formic acid. Sakami showed that one of the methyl carbons of acetone is oxidized to formate (12), and, since threonine probably is split to acetate and glycine (13), it is another potential source of formate.

The methyl group of methionine has been shown to be oxidized to carbon dioxide *in vivo* by Mackenzie and coworkers (14), employing C¹⁴-methyl-labeled methionine. The experiments reported here show that formate is formed *in vitro* from both methionine and choline and also from glycine and serine.¹

In the present work, ethionine, which has been shown to inhibit the transfer of methionine sulfur to cysteine sulfur (15), was employed with the aim of inhibiting the formation of formate from methionine and in order to secure further insight on the process of the synthesis of serine from glycine.

EXPERIMENTAL

Rats of the Long-Evans strain, weighing 170 to 200 gm., were employed in the present experiments. The sex and length of fasting periods are given in Table I. In certain experiments, ethionine was employed as a metabolic antagonist of methionine. The procedures employed in administering this substance are given as foot-notes to Table I.

The preparation of the liver slices and the methods for the isolation and

¹ Indication also of the formation of labeled formaldehyde from the methyl groups of methionine and choline and from the α - and β -carbons of glycine and serine respectively was obtained by adding carrier formaldehyde to incubated preparations. The isolation was performed by steam distilling the formaldehyde into dimedon solution and recrystallizing the methylenebis methone formed to constant specific radioactivity. These experiments are not reported here because of the possible contamination by coprecipitation of a radioactive contaminant. Experiments are now under way to prove rigidly the reliability of the method under the conditions of the experiments.

identification of serine and of formate and for the counting of these compounds have been previously described (2). The formate was determined

TABLE I
Production of Formate from Methionine

2.0 mg. of DL-methionine, labeled with C¹⁴ in the methyl group and having 191,000 c.p.m. (1.43 μ c.), were added to the medium to make a final concentration of 1.2 mm per liter.

Experiment No.*	Sex	Time of fasting	Treatment of animals	Addition to medium	Formate	
					Activity	Recovered counts†
		<i>hrs.</i>			<i>c.p.m.</i>	
1a	F.	35			1788	0.94
1b‡	"	35			174	0.09
2a	"	36			1338	0.70
2b	"	36		25 mg. ethionine	1128	0.59
3	"	36	Ethionine§		288	0.15
4	"	36	Ethionine-methionine		1788	0.94
5a	M.	27			2172	1.14
5b	"	27		60 mg. ethionine	504	0.26
6	"	27	Ethionine¶		468	0.24
7	"	27	Ethionine-methionine		2568	1.34
8		Boiled tissue			72	0.04

* Each experiment number refers to tissues from a particular animal.

† Recovered counts = $\frac{\text{specific activity of formate} \times \text{carrier added}}{\text{total counts added}} \times 100$.

‡ Experiment run under nitrogen.

§ 200 mg. of DL-ethionine in 0.9 per cent saline were injected intraperitoneally in four doses totaling 8 ml. during a period of 4 hours. The animal was killed 24 hours after the injection of the first dose. The female rats under this treatment had a visible fatty liver and increased fat in the liver (15).

|| 200 mg. of DL-ethionine plus 184 mg. (equimolar) of DL-methionine in saline were injected in the same way as above.

¶ These animals were killed immediately after the 4 hour treatment. The male rats under this 4 hour treatment had no visible fatty liver and no increased fat in the liver. The control rats were injected intraperitoneally with an equal volume of physiological saline. The authors are grateful to Dr. E. Farber for injecting the rats and for his observations on these animals.

by steam distillation and then by oxidizing it to carbon dioxide with mercuric oxide. In all experiments, about fifteen slices (750 mg., wet weight) were added to 10 ml. of Krebs-Ringer phosphate buffer, pH 7.4.

Both the C^{14} -methyl-labeled methionine² and the C^{14} -ethyl-labeled ethionine were synthesized by Tarver according to published methods (16, 17). The synthesis of β - C^{14} -labeled serine was developed by Levine and Tarver³ (18). The C^{14} -*N*-methyl-labeled choline chloride was synthesized by an unpublished method developed by F. C. Christensen and B. M. Tolbert of the Radiation Laboratory, University of California.³

Results

Formation of Formate from Methyl Group of Methionine

Table I shows clearly that the methyl group of methionine is transformed to formate. The process is inhibited in the ethionine-treated animals and by ethionine *in vitro* (when 60 mg. of ethionine are used).⁴ The inhibition *in vivo* is reversed by the simultaneous injection of methionine.

Ethionine has been found to inhibit the conversion of methionine sulfur to cysteine sulfur and to inhibit the uptake of methionine- S^{35} into the protein molecule (15). These inhibitions are reversed by methionine (15). The inhibition by ethionine of formate formation and the reversal by methionine, shown in Table I, are further indications that ethionine is a specific antagonist of methionine.

The production of formate from methionine is approximately equal to that produced from the α -carbon of glycine under the same conditions (2) and again, like the latter, apparently is an aerobic reaction, being decreased greatly under nitrogen.

It has been shown (2) that formate and glycine react, *in vitro*, to form serine; it was desirable, therefore, to test whether the methyl group of methionine could replace formate. Also, it follows, if this reaction does occur, that the incubation of α -labeled glycine, in the presence of inactive methionine, should result in a reduction in the per cent radioactivity in

² A paper chromatogram of the synthetic C^{14} -methyl-labeled methionine yielded two ninhydrin-reacting spots of approximately equal radioactivity (methionine and methionine sulfoxide) and a non-ninhydrin-reacting spot which accounted for about 10 per cent of the radioactivity. We are greatly indebted to Dr. Rosmarie Ostwald for the chromatographic analysis.

³ The authors are extremely grateful to Professor H. Tarver and Dr. B. M. Tolbert for furnishing the above C^{14} -labeled compounds.

⁴ The unpublished experiments of E. Farber and P. Siekevitz show that the 4 hour ethionine treatment has no effect on the oxygen consumption of the excised liver slices, while the 24 hour treatment gave a decrease of 30 to 50 per cent. The addition of ethionine *in vitro*, at levels corresponding to the 25 mg. addition described in the text, gave no effect on the oxygen consumption. Therefore, the results *in vivo* are probably not due to decreased oxygen consumption, while the addition of ethionine *in vitro* might decrease the respiration, but this is unlikely.

the β -carbon of serine from that found when only radioactive glycine is employed. Table II confirms these expectations. The formation of radioactive cystine was also investigated but none was found.

Although it is clear that the methyl group of methionine produces formate which can react with glycine to form serine, the data of Table II indicate that the relationships involved are not simple. It is seen that methionine, like formate and acetate, increases the over-all formation of labeled serine from glycine; at the same time the percentage of radioactivity in the β -carbon of serine is cut down with respect to the over-all

TABLE II

Production of Serine from Glycine and from Methionine

2.0 mg. of DL-methionine, labeled with C^{14} in the methyl group and having 191,000 c.p.m. (1.43 μ c.), were added. In the glycine experiments, 1.0 mg. of glycine, labeled with C^{14} in the α -carbon and having 277,000 c.p.m. (2.07 μ c.), was added. The final concentration in both cases was 1.2 mm per liter. Carrier serine was added at the end of the incubation run, and in the cystine isolation carrier cystine was added. The serine was isolated and degraded as previously described (2). The cystine was recrystallized twice from water and the copper mercaptide of cysteine was made. This derivative was then hydrolyzed, recrystallized once from water-alcohol, and counted as such. The livers were from animals fasted for about 24 hours.

Experiment No.	Radioactive substrate	Addition to medium*	Recovered counts of serine	
			Total	β -C
1†	Methionine		0.53	0.43
2†	"	Glycine	1.21	1.17
3	Glycine	Methionine	8.2	0.95
4†	"		4.2	1.3
5†	"	Formate	7.9	1.3
6†	"	Acetate	10.4	1.2

* In amounts equimolar with the radioactive substrate.

† Cystine was isolated as described above, but no radioactivity could be detected.

‡ These experimental results from Siekevitz and Greenberg (2) are presented here for comparison.

radioactivity of the serine. It is not clear whether this represents a real increase, or whether the formation of glycine from unlabeled sources is reduced, thus decreasing the dilution of the added radioactive glycine. It is hard to conceive of formate, methionine, or acetate inhibiting the endogenous synthesis of glycine. Since methionine gives rise to formate, it might act via formate, and, thus, formation of formate might be the limiting factor in the synthesis of serine from glycine.

The effect of acetate, however, is different. We have tested the capability of both carboxyl- and α -labeled acetate to form formate under

various conditions with inconclusive results. Radioactive formate was obtained only from carboxyl-labeled acetate, only in the fasted rat, and only in the presence of glutamine, but the reaction is highly erratic.⁵ In this regard, Schulman, Buchanan, and Miller have recently found that carboxyl-labeled acetate does not contribute to the ureide carbons of uric acid in the pigeon (19), and this has been confirmed by Elwyn and Sprinson (20) and by Valentine, Gurin, and Wilson in the rat (21).

Formation of Formate from Methyl Groups of Choline

The results of the tests with choline on the formation of formate⁶ from the labeled methyl group are given in Table III.

The amount of formate formed is only about one-fourth the quantity obtained from the methyl group of methionine, at least in air. Under nitrogen, on the contrary, formate is produced from choline at a greater rate than it is produced anaerobically from methionine, and even at a greater rate than it is produced aerobically from choline. The reason for this is unknown. With choline, the inhibition by ethionine is much greater *in vitro* than *in vivo*, in contrast to the methionine experiments. The lack of inhibition by ethionine *in vivo* of the choline to formate step strongly suggests that the methyl group of choline is oxidized to formate directly, and not only via methionine. Experiments 8b and 8c in Table III show that added inactive formaldehyde cuts down the radioactivity of the formate, both aerobically and anaerobically, thus apparently serving as an intermediate in both these circumstances. Therefore, since, under nitrogen, the production of formate from choline is increased, it follows that oxygen inhibits either the oxidation of the methyl group to formaldehyde or the oxidation of the formaldehyde to formate.

In the ethionine-methionine-treated animal (Experiment 4, Table III) there is to be noted the rather startling result that there is a large increase in the production of formate over that of the control rat. A possible explanation is that, in the presence of excess methionine (in the ethionine-methionine-treated animals and in their excised liver slices), the demethylation of choline is inhibited so that more of the choline methyl is either oxidized directly or after conversion to betaine. Thus, methionine alone should stimulate the formation from choline, and in Experiment 5 this stimulation is shown, though its magnitude is not as great as that obtained in the ethionine-methionine-treated animal.

⁵ Unpublished data.

⁶ Since choline is probably decomposed to trimethylamine by the procedure used to isolate the formate, this compound was tested for its oxidation to CO₂ by mercuric oxide (the method used to oxidize the isolated formate to CO₂). It was not oxidized to CO₂ under these conditions.

*Effect of Ethionine on Production of Formate from Glycine and on
Formation of Serine from Glycine*

Evidence for the formation of formate from the α -carbon of glycine has previously been reported by us (2) and by Sakami (22). Experiments 1 through 6 of Table IV, on the effect of ethionine on the production of

TABLE III
Production of Formate from Choline

2.0 mg. of choline chloride, labeled with C^{14} in the methyl group and containing 209,000 c.p.m. (1.56 μ c.), were used. The livers of male rats, fasted 24 hours before sacrificing, were used except when noted.

Experiment No.*	Treatment of animal	Formate	
		Activity	Recovered counts
		c.p.m.	
1		380	0.20
2a		426	0.20
2b†		198	0.09
3	Ethionine‡	372	0.18
4	Ethionine-methionine§	2190	1.05
5	Methionine	888	0.43
6¶		66	0.03
7g**		1296	0.62
8a††		687	0.33
8b‡‡		240	0.12
8c†† ‡‡		318	0.15

* Each experiment number refers to tissues from a particular animal.

† 60 mg. of DL-ethionine added to the flask.

‡ Treatment described in foot-notes to Experiments 3 and 6, Table I.

§ Treatment described in foot-note to Experiment 4, Table I.

|| 184 mg. of DL-methionine in 0.9 per cent saline were injected intraperitoneally in four doses totaling 8 ml. during a period of 4 hours. The animal was sacrificed immediately after the 4 hour treatment.

¶ Boiled tissue.

** The female rat was fasted for 46 hours previous to sacrificing.

†† Experiment run in an atmosphere of 95 per cent N_2 -5 per cent CO_2 .

‡‡ At the beginning of the experiment, 1.0 mg. of formaldehyde was added in addition to the 1.2 mg. of formate normally added.

formate from glycine, were performed as a control prior to conducting experiments on the influence of ethionine on the degree of incorporation of the C^{14} of the methylene group of glycine into the β -carbon of serine. No effect was anticipated and the observed inhibition of the formation of formate from glycine by ethionine shown in Table IV was totally unexpected. No satisfactory explanation can be given at present.

Since both the methyl group of methionine and the α -carbon of glycine form the β -carbon of serine, probably via formate, it would be anticipated that in the ethionine-treated animals (following the conversion of the C^{14} of the methylene group of glycine to serine) the percentage of radioactivity in the β -carbon of serine with respect to total radioactivity would be increased, owing to the inhibition of the formation of unlabeled formate from methionine (Table I). The lack of any increase might be explained by the suggestion that methionine is not a direct formate donor

TABLE IV

Effect of Ethionine on Production of Formate from Glycine and on Conversion of Glycine to Serine

1.0 mg. of glycine, labeled with C^{14} in the α -carbon and containing 276,000 c.p.m. (2.07 μ c.), was added. The livers of male rats, fasted 24 hours before sacrificing, were used. 1.2 mg. of formate were added in all cases except Experiments 7, 8, and 9

Experiment No.		Formate		Recovered counts of serine	
		Activity	Recovered counts	Total	β -C
		c.p.m.			
1		1956	0.71		
2	Ethionine*	1063	0.38		
3	Ethionine-methionine†	1008	0.36		
4‡		1152	0.42		
5	Ethionine*	1194	0.43		
6	Ethionine-methionine†	1314	0.47		
7§				5.0	1.1
8	Ethionine*			4.3	1.1
9	Ethionine-methionine†			4.2	1.4

* Treatment described in foot-notes to Experiments 3 and 6, Table I.

† Treatment described in foot-note to Experiment 4, Table I.

‡ 60 mg. of DL-ethionine added to flask.

§ Animal injected intraperitoneally with saline.

for the β -carbon of serine. The immediate donor, other than glycine, might be choline, as is indicated by the lack of *in vivo* inhibition by ethionine of the choline to formate reaction and by the increase of the formate under anaerobic conditions. It has been postulated that the extra glycine source of the β -carbon of serine is more active anaerobically than aerobically (2), and choline is the only known compound at present which fits into this postulation.

Production of Formate from Serine

It has been shown that the methyl groups of methionine and choline and the α -carbon of glycine (2) yield formate. Experiments were, there-

fore, performed to determine whether the β -carbon of serine produces formate, as envisaged by Shemin (23). The results are recorded in Table V. The formation of formate from serine follows the pattern observed with glycine and methionine.

Formate production decreases anaerobically as expected. In one series of experiments inactive formaldehyde was added to radioactive serine, and the formate was caught. The results (Table V, Experiments 1c and 1d) show that, whereas in air the added formaldehyde had no effect, under nitrogen it decreased the radioactivity of the formate formed, pre-

TABLE V
Production of Formate from Serine

In these experiments 1.4 mg. of DL-serine (concentration 1.2 mM per liter), labeled with C^{14} in the β -carbon and having 440,200 c.p.m. (3.29 μ c.), were used. The livers were mainly of male rats fasted 24 hours previous to sacrifice. Exceptions to this are noted in the table. 1.2 mg. of formate were added at the beginning to trap the formate formed and 60 mg. as carrier at the end of each experiment.

Experiment No.*	Atmosphere	Formate	
		Activity	Recovered counts
		c.p.m.	
1a	Air	15,096	3.43
1b	N ₂	3,192	0.73
2a†	Air	18,046	4.10
2b†	N ₂	7,260	1.65
1c‡	Air	15,252	3.46
1d‡	N ₂	1,416	0.32
3§		348	0.066

* Each number refers to tissues from a particular animal.

† Animals fasted 16 hours before sacrifice.

‡ To each flask, in addition to the 1.2 mg. of formate, 1.0 mg. of formaldehyde was added at the beginning.

§ Boiled tissue.

sumably by dilution. Thus, formaldehyde appears to act as an intermediate under nitrogen but not as one under air.

DISCUSSION

Formation of formate in biological systems has now been shown to occur from the labile methyl groups of methionine and choline and from the α -carbon of glycine and β -carbon of serine. Oxidation of the methyl group of sarcosine, which forms formaldehyde (4, 5), probably continues on to formate. The relationship of formaldehyde to formate is difficult to assess. While it appears probable that, at least in some instances, formaldehyde production precedes the formation of formate, a great deal

more experimental work is required to clarify the facts. We have found that in the system which yields formate from glycine radioactive formate is not reduced to give radioactive formaldehyde, in air or in nitrogen.⁵

Production of formate, it may be postulated, is one of the chain of steps in the oxidation of labile methyl groups and of certain other carbon entities to carbon dioxide. The oxidation of the methyl group of methionine to carbon dioxide has been demonstrated by Mackenzie *et al.* (14). The authors have observed that practically all the radioactivity of formate added to rat liver slices can be accounted for in the evolved carbon

TABLE VI

Formation of Radioactive Methionine from Homocysteine and Radioactive Formate in Vitro

To the incubation mixture there were added 0.6 mg. of labeled formate with a specific activity of 2.2 μ c. (294,000 c.p.m.) and 1.8 mg. of DL-homocysteine. About 750 mg., wet weight, of rat liver slices were added to 10 ml. of Krebs-Ringer phosphate buffer, pH 7.4. At the end of the 3 hour run 800 mg. of DL-methionine were added and the methionine was recrystallized from water to constant specific activity, usually six or seven recrystallizations. The methionine was then demethylated by the method of Baernstein (25), the methyl iodide being caught in cold absolute alcohol. The *S*-methylisothiouraea picrate derivative of the methyl iodide was prepared as described by Brown and Campbell (26). The melting point of this compound (uncorrected) in Experiment 2a was 216–217° and in Experiment 2b, 218–220°. The reported value is 224° (26).

Experiment No.*	Atmosphere	Total		Methionine methyl C		Carboxyl C	
		Activity	Recovered counts	Activity	Recovered counts	Activity	Recovered counts
		c.p.m.		c.p.m.		c.p.m.	
1a	N ₂	2,320	0.79			0	0.0
1b	Air	5,600	1.93			1440	0.49
2a	N ₂	480	0.19	80	0.06	360	0.12
2b	Air	10,160	3.56	5520	1.92	1040	0.35

* Each experiment number refers to a different animal.

dioxide at the end of 3 hours.⁵ Some of the formate may be diverted from further oxidation and be utilized for synthetic purposes in the organism, as in the synthesis of serine and the purines.

The partial oxidation of labile methyl groups may have no great significance for transmethylation in the animal organism. However, we have confirmed the observation of Welch and Sakami (24) that formate is reduced to the methyl group of methionine *in vivo* and *in vitro*. Table VI gives the result. Filter paper chromatograms on the methionine isolated from the radioactive formate showed that any contamination, if present, would be less than 1 per cent. This contamination is much too

low to account for all the radioactivity in the methionine. We are unable to account for all the activity of the methionine in Experiment 2b. The fixation of formate *in vivo* into the methyl carbon of methionine may not be very high, for du Vigneaud and coworkers (27) have shown an 8 per cent synthesis of labile methyl groups in 3 weeks. Under different experimental conditions this may be much higher, as envisaged by Stekol *et al.* (28).

SUMMARY

1. The methyl group of methionine has been shown to give rise to formate and can serve as a source of the β -carbon of serine. The former reaction is inhibited by ethionine *in vivo* and *in vitro* and is reversed by methionine.

2. The methyl group of choline also gives rise to formate. In this case, ethionine inhibits the production of formate *in vitro* but not *in vivo*. Administration of methionine increases the production of formate over that obtained in the control animals, possibly by a choline-sparing action.

3. The β -carbon of serine has been shown to form formate. The administration of ethionine inhibits the *in vitro* production of formate from glycine, but does not inhibit the *in vitro* synthesis of serine from glycine. The inhibition is not reversed by the *in vivo* injection of methionine.

4. Formate production from the above four compounds, except for choline, is greater in air than in nitrogen.

5. It has been postulated that the *N*-methyl carbons of choline and the α -carbon of glycine are the specific formate donors for the β -carbon of serine.

6. It has been confirmed that formate is reduced, possibly not via formaldehyde, to become the methyl group of methionine.

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ENZYMATIC CLEAVAGE OF THIOETHERS*

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In a previous publication, the purification of the enzyme responsible for the cleavage of cystathionine was reported (1). It was emphasized that in the course of the purification the activity toward cysteine was retained and in no case was there a fractionation of activity toward the two substrates. In this report, certain aspects of the specificity of the preparation and additional evidence for the identity of the enzyme acting upon cysteine and cystathionine are reported. In addition, a brief study of the activation of the enzyme by metal ions is summarized.

EXPERIMENTAL

The enzyme was prepared from liver tissue of rats as has been previously described (1). It has been found that when the centrifuged homogenate was dialyzed against physiological saline overnight at 0° before the treatment with heat the material could be heated to 60° for 10 minutes without appreciable loss of activity; otherwise, the preparation was as in the previous publication (1). It is to be noted, however, that the activities reported here are about one order of magnitude greater than those previously reported. This greater activity is due to the addition of glutathione to the solution of enzyme; in the presence of glutathione, activity remains unchanged for several weeks at 2°. The methods of analysis were as follows: Cysteine was determined by the method of Sullivan and Hess (2), soluble total sulfides by the method of Brand, Cahill, and Kassell (3), and the volatile sulfides by iodometric titration of the materials trapped in cadmium acetate (1). In the latter method, very poor results were observed at low levels. Hydrogen sulfide was determined by the reaction involving the formation of methylene blue (4).

The compounds used in this study were prepared by Dr. M. D. Armstrong and Mr. J. D. Lewis of this laboratory; improved techniques of preparation and purification will be described in a subsequent report.

Specificity of Thionase¹ Preparations—A purified preparation of thionase

* These studies were supported by a grant from the United States Public Health Service.

¹ Thionase is used to designate the activity toward compounds containing bivalent sulfur; no implication as to homogeneity is intended. Preparations of the

was tested with the compounds listed in Table I as substrates. From the results given here it is apparent that the preparation will attack many compounds containing the L-cysteine moiety. Cysteine was identified as a product in the cleavage of L-cystathionine and djenkolic acid. Hydrogen sulfide was the product of cleavage of cysteine, cysteine methyl ester, and cysteinylglycine. Since cysteine could not be detected as a

TABLE I
Specificity of Thionase

0.090 mm of substrate was incubated for 1 hour with 2 mg. of glutathione in 10 ml. of 0.02 M phosphate buffer, pH 7.3. 0.0007 mg. of protein nitrogen was present in each ml. of digest.

Compound	Cleavage	Cysteine	Soluble sulfide	Volatile sulfide
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
L-Cysteine.. . . .	35			35*
L-Cystathionine.. . . .	74	52		22*
L-Allocystathionine.. . . .	39		39	
S-Methyl-L-cysteine.. . . .	21	0	0	21†
S-Ethyl-L-cysteine.. . . .	16	0	0	16†
S-Propyl-L-cysteine.. . . .	10	0	3‡	7†
S-Butyl-L-cysteine.. . . .	2	0	2‡	0
S-Carboxymethyl-L-cysteine	7	0	6§	1
S-Carboxyethyl-L-cysteine.. . . .	1	0	1	0
S-Carboxypropyl-L-cysteine.. . . .	0	0	0	0
Djenkolic acid.... .	42	20		22*
L-Cysteine methyl ester.. . . .	71	0		71*
L-Cysteinylglycine.. . . .	31	0		31*

* Hydrogen sulfide.

† Volatile mercaptans, not hydrogen sulfide.

‡ Extractable with organic solvents from neutral, acid, or alkaline solution.

§ Extractable with organic solvents from acidic solution.

|| Determined by the method of Nakamura and Binkley (7).

product of the cleavage of the latter two compounds, it is presumed that the cleavage occurred without preliminary hydrolysis to cysteine. Cysteine methyl ester appears to be a better substrate than cysteine.

The cleavage of S-alkyl derivatives of cysteine, when observed, brought

enzyme from rat liver, bacteria, dried yeast, and from kidney tissue of the pig have been found to have different specificity relationships. In the preparations from tissues other than rat liver, glutathione must be added before any activity is observed. Glutathione has been included in the tests with the preparations from liver tissue of rats so that the data obtained with different preparations of the enzyme will be comparable. Cyanide or cysteine will not replace the glutathione in the activation of the preparations.

about the formation of volatile mercaptans. Methyl, ethyl, and propyl mercaptans were identified as their mercury derivatives (5).

In agreement with other investigators (6), we have found that djenkolic acid, when mixed with the diet or when injected intraperitoneally, will not serve in lieu of cysteine for growth of the white rat. Nevertheless, djenkolic acid is cleaved by the enzyme with the production of cysteine as determined by the method of Sullivan and Hess (2) or by that of Nakamura and Binkley (7). The only explanation we can offer is that djenkolic acid is not sufficiently soluble to be absorbed from the intestine or from the peritoneum.

Since glutathione and *N*-benzoylcysteine were not attacked by the enzyme, whereas cysteine methyl ester and cysteinylglycine were attacked, it may be suggested that a free amino grouping but not a free carboxyl grouping is essential for the activity of the enzyme. This is borne out by the contrast of cystathionine, which is perhaps the best substrate for the enzyme, and *S*-carboxypropylcysteine which was not attacked to a detectable extent. The data to date on the specificity of thionase are summarized in Table II.

Competitive Inhibition of Activity of Thionase Preparations—The various substrates were tested, in so far as the methods of analysis permitted, as inhibitors of the cleavage of L-allocystathionine. Certain of the results are reported in Fig. 1. L-Allocystathionine is an ideal substrate for such studies, since the product, D-homocysteine, is not attacked by the enzyme and no volatile products are formed. It is possible, therefore, to distinguish between the products from the various substrates used as inhibitors and the product from L-allocystathionine. The volatile products were removed by aeration and the products soluble in organic solvents were removed by extraction with chloroform. Cysteine and total sulfides were determined as the disulfides by the methods described above and homocysteine was calculated as the difference of the two methods. It is seen that cysteine and the derivatives of cysteine were competitive inhibitors of the cleavage of L-allocystathionine. The inhibition obtained with the *S*-alkyl derivatives was predictable upon the basis of the relative rates of cleavage of the compounds. With these derivatives, it is reasonable to assume that the rate of cleavage is regulated by the rate of formation of the enzyme-substrate complex (first order reaction rates were observed with the *S*-alkyl substrates at the concentrations allowed by their solubility). With the *S*-carboxyalkyl derivatives and with cysteine, the inhibition was found to bear no relationship to the relative rates of cleavage; all these compounds inhibited to a much greater extent than they did in competition for the formation of an enzyme-substrate complex. Thus, the presence of an acidic grouping attached to the sulfur (H as in cysteine

TABLE II

Summary of Specificity of Thionase

The bond broken in the cleavage is indicated by the dotted line. The values for the rate of cleavage represent sulfur released as compared with cystathionine as 100.

Compound	Formula	Relative rate of cleavage	Reference
L-Cysteine.....	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{SH}$	50	Present paper
S-Methyl-L-cysteine.....	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_3$	30	"
S-ethyl-L-cysteine.....	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2\text{CH}_3$	20	"
S-Propyl-L-cysteine.....	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2\text{CH}_2\text{CH}_3$	10	"
S-Butyl-L-cysteine.....	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$	1	"
S-Carboxymethyl-L-cysteine	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2\text{CH}_2\text{COOH}$	10	"
S-Carboxyethyl-L-cysteine	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$	5	"
S-Carboxypropyl-L-cysteine	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$	0	"
L-Lanthionine.....	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CHNH}_2-\text{COOH}$ (L, L)	100	*
L-Cystathionine.....	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{CHNH}_2-\text{COOH}$ (L, L)	100	Present paper (11)
L-Alloecystathionine	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{CHNH}_2-\text{COOH}$ (L, D)	50	" (11)
D-Cystathionine....	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{CHNH}_2-\text{COOH}$ (D, D)	10	(11)
D-Alloecystathionine..	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{CHNH}_2-\text{COOH}$ (D, L)	50	(11)
Dienkolic acid	$\text{HOOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{CH}_2-\text{CH}_2-\text{CHNH}_2-\text{COOH}$	100	Present paper
L-Cysteine methyl ester	$\text{CH}_3\text{OOC}-\text{CHNH}_2-\text{CH}_2-\text{S}-\text{H}$	100	"
L-Cysteinylglycine.....	$\text{HOOC}-\text{CH}_2\text{NH}-\text{OC}-\text{CHNH}_2\text{CH}_2-\text{S}-\text{H}$	50	"

* Du Vigneaud, V., Anslow, W. P., Jr., and Binkley, F., unpublished observations.

and *S*-carboxyalkyl groupings) was observed to increase the affinity of the substrate toward the enzyme but to retard the cleavage. One possible physiological implication of these findings is that the concentration of cysteine may be the regulatory factor in the cleavage of cystathionine to

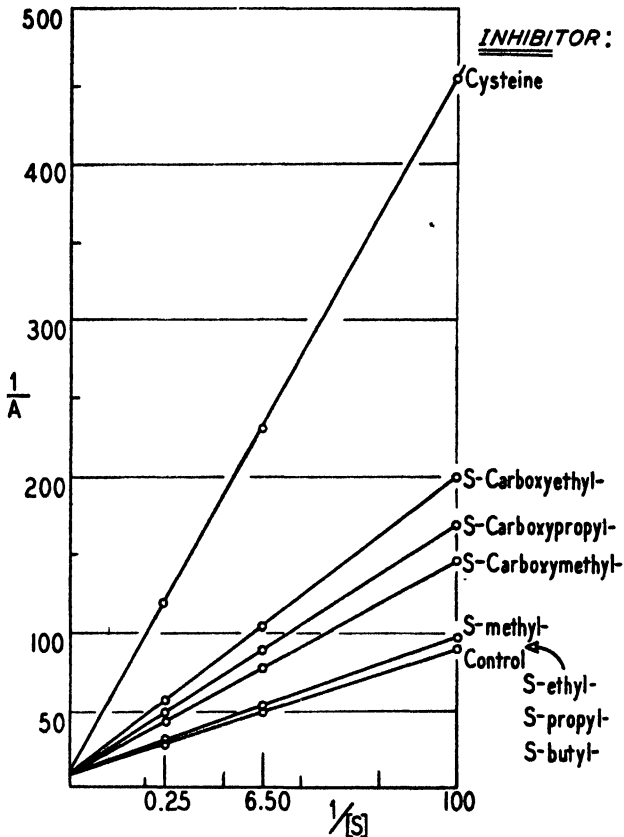


FIG. 1. Competitive inhibition of thionase. L-Allocystathionine and 0.001 mm per ml. of inhibitor were incubated in a total volume of 10 ml., 0.01 M barbiturate buffer, pH 7.8. 0.0003 mg. of protein nitrogen was present in each ml. of digest and the time of incubation was 30 minutes at 37°. Activity is expressed as mm per minute per liter and substrate concentration as mm per liter.

cysteine and, thus, the conversion of methionine to cysteine by the intact animal.

When the activity of the enzyme toward L-cysteine and L-cystathionine was tested in the presence of various substrates, all the materials were found to inhibit in a competitive manner; some typical results with L-cystathionine, L-allocystathionine, and L-cysteine are summarized in

Table III. It has been demonstrated that, throughout this series of compounds, competitive inhibition occurs; it is hardly conceivable that such results could have been obtained unless one enzyme were responsible for the cleavage of L-cysteine and L-cystathionine, as well as for the cleavage of the substrates not known to be of physiological importance. It is the suggestion of the author that "thionase" should replace the numerous previous names (cysteine desulfurase, cysteine desulphydrase, cystathionase, etc.) for this enzyme.

Activation by Metal Ions—Crude preparations of thionase were found to be activated by the addition of magnesium or zinc ions (1, 8, 9). It was

TABLE III
Competitive Inhibition of Thionase

0.01 mm of substrate was incubated with 0.02 mm of inhibitor in 10 ml. of 0.01 M barbiturate buffer, pH 8.0, for 30 minutes. 0.0003 mg. of protein N was present in each ml. of digest. When L-cystathionine was the substrate, cysteine was determined by the method of Sullivan and Hess (2); with L-allo-cystathionine as the substrate, homocysteine was determined by the method of Brand, Cahill, and Kassell (3), and with L-cysteine as the substrate, hydrogen sulfide was determined as methylene blue (4). Substrate-inhibitor pairs were selected in a manner such that interference in the analytical procedures was avoided.

Inhibitor	Substrate		
	L-Cystathionine	L-Allo-cystathionine	L-Cysteine
	<i>per cent inhibition</i>	<i>per cent inhibition</i>	<i>per cent inhibition</i>
S-Methylcysteine.....	12	7	8
S-Carboxymethylcysteine.....	35	34	22
S-Carboxyethylcysteine..	45	55	22
S-Carboxypropylcysteine	33	51	15
L-Allo-cystathionine ..	29		10
L-Cysteine.....		80	

surprising to find that the purified thionase was either unaffected or was inhibited by the addition of magnesium ions. These results are described in Table IV. It is possible that the enzyme was saturated with magnesium and that additional amounts would either be without effect or would be inhibitory. It was impossible to demonstrate magnesium in the enzyme, but it must be emphasized that the methods might not be sensitive enough to permit the demonstration of amounts of magnesium sufficient for activation of the enzyme. Therefore, a study with various inhibitors was undertaken and certain of the results are summarized in Table IV. It is evident that the activity of the enzyme was reduced by the addition of phosphate, cyanide, and glutathione. Evidence similar to this has been

TABLE IV

Metal Activation of Thionase

L-Allocystathionine, 2×10^{-3} mm per ml., was incubated with the enzyme in 0.01 M barbiturate buffer, pH 7.8, for 30 minutes and the product of cleavage was determined by the method of Brand, Cahill, and Kassell (3).

Enzyme preparation	Concentration of activator	Control activity <i>per cent</i>
Crude extract	10^{-3} M Mg^{++} , 2 hrs. preincubation	225
	10^{-3} " " 2 hrs. preincubation	152
Dialyzed* crude extract	10^{-3} " " 2 hrs. preincubation	130
" " "	10^{-3} " " 2 hrs. preincubation	100
Purified enzyme	10^{-3} " " 2 hrs. preincubation	77
" "	10^{-3} " "	100
" "	10^{-1} " phosphate, pH 7.3*	57
" "	10^{-2} " cyanide, pH 7.6*	17
" "	10^{-3} " glutathione	22

* Dialyzed overnight against 0.1 M NaCl.

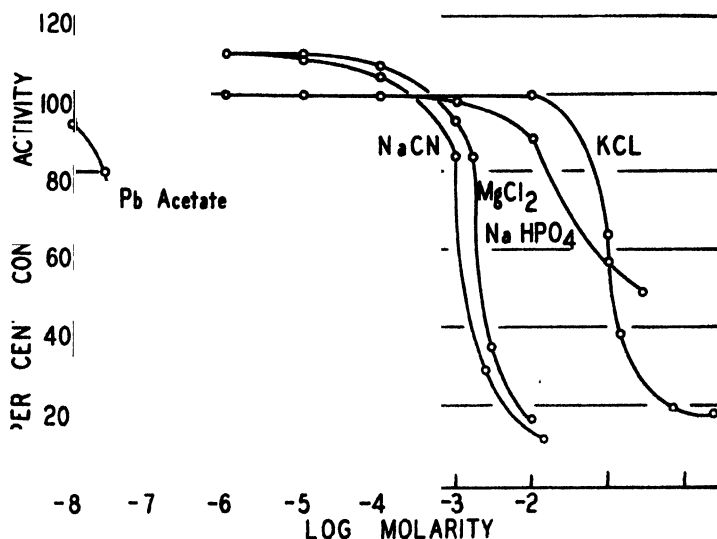


FIG. 2. Ionic inhibitors of thionase. L-Allocystathionine, 0.004 mm per ml., was incubated with an amount of enzyme represented by 0.0007 mg. of protein nitrogen per ml. of digest. The enzyme was allowed to stand with the various materials for 30 minutes before addition of the substrate.

presented for the concept that carboxypeptidase is a magnesium-protein complex (10).

When certain extraneous materials such as sodium chloride, potassium

chloride, and sodium acetate were tested in a similar fashion, it was found that these materials inhibited the enzyme much as did cyanide or glutathione. Certain comparative studies are summarized in Fig. 2. It is seen that lead acetate, sodium cyanide, magnesium chloride, and potassium chloride inhibited in a similar manner; these curves are those for a theo-

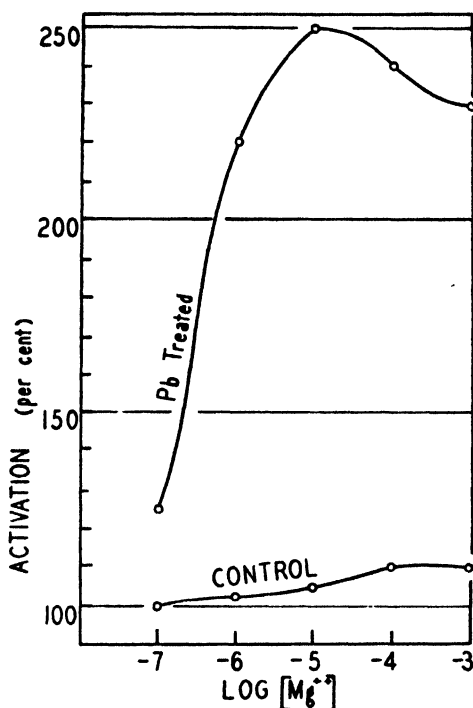


FIG. 3. Reversal of inhibition by lead ions. Purified thionase was mixed with a solution of lead acetate and barbiturate buffer, pH 7.8. The final concentration of lead was 5×10^{-7} M in the digest. The solutions were incubated 30 minutes before the addition of L-alloeystathionine (0.004 mm per ml.). The time of incubation was 30 minutes at 37°. The control activity of the enzyme treated with lead was 20 per cent of that of the untreated material (lower curve).

retical inhibition in which 2 molecules of inhibitor are combined with one active site of the enzyme (10). Sodium fluoride, sodium acetate, and sodium chloride behaved in a similar manner; the curves fell, in the order indicated, between sodium cyanide and potassium chloride. Sodium citrate was less effective than phosphate as an inhibitor but gave a similar curve, corresponding to a combination of 1 molecule with one active site of the enzyme. It is hardly conceivable that potassium chloride, sodium chloride, and sodium acetate are acting as inhibitors by combination with magnesium ions.

Some clarification of the situation was obtained in an accidental manner. It was found that when magnesium ions were added to a preparation that had been inactivated by the addition of lead ions considerable reactivation could be demonstrated; such a study is summarized in Fig. 3. It is to be noted that at the higher levels of magnesium ions inhibition was observed as in the previous study. Nevertheless, the addition of magnesium ions was found to reverse the inhibition by lead ions. It would appear possible, therefore, that the activation of crude preparations of thionase by magnesium ions is dependent upon the presence of inactivating ions. The enzyme was inhibited by cupric or ferric ions added in the range of 10^{-7} to 10^{-6} M; these inhibitions were reversed by the addition of magnesium chloride. It is probable that either cupric or ferric ions at such low levels may be present in the crude extracts of tissues. The inhibitions by lead, cupric, or ferric ions were not reversed by the addition of glutathione or cyanide, although these materials would prevent the inhibition if present at the time of addition of the inhibitor.

These results are not to be considered as evidence that all activations of enzymes by metal ions are concerned with the reversal of inhibitions by other metals, but a note of caution would appear to be in order. It is apparent that activation by the addition of a metal ion, enhanced activation by preincubation with a metal ion, or inhibition by agents that combine with metal ions cannot be accepted as proof of the participation of a metal in an enzymatic process.

SUMMARY

The specificity of preparations of the enzyme responsible for the cleavage of cystathionine and cysteine toward derivatives of cysteine has been considered in some detail. A free amino grouping on the L-cysteine moiety was found to be essential for the activity of the enzyme; a free carboxyl grouping was not essential. The substituent on the sulfur atom may vary from hydrogen (cysteine) to γ -carboxy- γ -aminopropyl (cystathionine). When the length of the carbon chain of alkyl and carboxyalkyl groupings substituted for the hydrogen of the cysteine was increased, decreasingly lower activity was observed.

The activation of purified preparations of the enzyme by magnesium ions could not be demonstrated unless inhibitory ions were added. It was concluded that the activation of crude preparations of the enzyme was dependent upon the presence of inhibitory ions.

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INCORPORATION IN VITRO OF LABELED AMINO ACIDS INTO BONE MARROW CELL PROTEINS*

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Nearly all experiments on the incorporation of labeled amino acids into tissue proteins *in vitro* have been done on tissues whose cell structure has been partially or completely disintegrated, e.g. tissue slices, segments, or homogenates. Since cell destruction reduces or abolishes the uptake of labeled amino acids (1), it seemed worth while to carry out studies on intact cells *in vitro*. Bone marrow cells were found to be suitable for this purpose. The labeled amino acids used were glycine-1-C¹⁴, L-leucine-1-C¹⁴, L-lysine-1-C¹⁴, and L-lysine-6-C¹⁴.

Preparation of Marrow Cells

In preliminary experiments it was found that the bone marrow cells of guinea pig, rabbit, and rat were all very active, but too many guinea pigs or rats were needed to obtain a convenient amount of bone marrow cells to work with. All the results reported here were obtained with rabbit marrow cells. Three or four rabbits were usually needed for an experimental run. The rabbits were obtained commercially; they were of both sexes and in normal nutrition. They were killed by a blow on the head and bled. The humerus, ulna, femur, and tibia were removed as quickly as possible; usually it took three workers 45 minutes to remove and clean these bones from four rabbits. Since it was too inconvenient to remove the bone aseptically, after the bones were removed (without any aseptic precautions), they were immersed in ice-cold 0.1 per cent phenol for 5 minutes, then in ice-cold 70 per cent ethanol for 5 minutes, and then transferred to ice-cold sterile 0.9 per cent NaCl. The ends of a bone were sawn off and the marrow was pushed out of the shaft and scraped out of the ends with a steel knitting needle. The marrow was collected in Krebs-Henseleit Ringer's solution (2), then passed through six layers of cheese-cloth to break up the lumps, suspended in 10 volumes of the Ringer's solu-

* This work was carried out under the joint sponsorship of the United States Atomic Energy Commission and the Office of Naval Research. The C¹⁴ used was supplied by the Carbide and Carbon Chemicals Corp., Oak Ridge, Tennessee, and obtained on allocation from the United States Atomic Energy Commission.

tion and centrifuged in a refrigerated centrifuge at 2° at about $2000 \times g$ for 10 minutes. The sedimented cells were suspended in about 10 ml. of the Ringer solution and the pH was adjusted to 7.8.

The equipment used in the collection of the marrow cells was sterilized before use. There was a possibility that the incorporation of labeled amino acids was due to bacterial action. We checked the degree of bacterial infestation by plating out reaction mixtures which had been incubated for 6 hours at 38° . In no case did we find more than 50 colonies per ml. of reaction mixture; usually there were none. We compared the results in reaction mixtures with and without additions of 1000 units of penicillin (G) per ml. We had found earlier that, with this concentration of penicillin, reaction mixtures with an initial heavy bacterial infestation became nearly or completely bacteria-free at the end of a 6 hour experimental run. No difference was found in the uptake of labeled amino acids with and without added penicillin. As an additional precaution against bacterial action penicillin was used in most of the experiments.

Labeled Amino Acids

Glycine labeled with C^{14} in the carboxyl group was synthesized according to the method of Sakami *et al.* (3). The synthesis and resolution of L-leucine and of L-lysine labeled with C^{14} in their carboxyl groups (4) and of L-lysine in position 6 (5) have been described previously.

The activity of the labeled amino acids used in the experiments was as follows: glycine-1- C^{14} , 13,500 c.p.m. per mg.; L-leucine-1- C^{14} , 6000 c.p.m. per mg.; L-lysine-1- C^{14} , 16,500 c.p.m. per mg.; L-lysine-6- C^{14} , 15,000 c.p.m. per mg.

Procedure

The solvent and the suspending fluid used throughout was Krebs-Henseleit Ringer's solution. The reaction mixtures were made up in 20 ml. beakers and incubated in the Dubnoff apparatus (6) at 38° under 95 per cent O_2 and 5 per cent CO_2 , except in a few cases in which the mixtures were anaerobic under 95 per cent N_2 and 5 per cent CO_2 . The incubation time was 4 hours unless otherwise stated. The volume was either 1.0 or 1.01 ml. and contained, except as noted, in addition to other components, 0.1 ml. of a solution of penicillin G containing 10,000 units per ml., 0.01 ml. of 0.4 M $CaCl_2$ solution, pH adjusted to 6.0, and 0.1 ml. of a solution of each amino acid. At the end of the incubation 4 ml. of water were added, the pH was measured, more water was added to a volume of 80 ml., and, then, with stirring, 20 ml. of 35 per cent trichloroacetic acid. After standing overnight at room temperature, the precipitated protein was centrifuged, washed, and dried and its radioactivity measured as previously described (7).

The results given are the average of duplicates; they did not vary more than ± 10 per cent from the average.

Results

Fig. 1 shows that the rates of incorporation of the three amino acids into the marrow cell protein did not slacken off greatly until after 3 hours.

Fig. 2 shows how the amount of labeled amino acid incorporated varied with the initial concentration of the amino acid. In all three instances the relationship was logarithmic until the initial amino acid concentration that gave maximum incorporation was reached. With higher concentrations the uptake gradually became less, especially with leucine. The marrow cells incorporated the labeled amino acids from concentrations as low as those in the blood, and, in this range, the rate of incorporation varied most with the initial concentration of the amino acid.

The maximum amounts of glycine, leucine, and lysine incorporated on the basis of the observed radioactivity of the proteins were 5.5, 8.9, and 5.4 $\text{mm} \times 10^{-3}$ per gm. (dry weight) of protein respectively in 3 hours. Over this time interval the average rates were only a little less than the initial rates (Fig. 1).

Only about one-quarter of the counts in the protein after incubation with labeled glycine was due to glycine incorporated as such into the proteins; with leucine or lysine practically all the counts in the protein were due to the amino acids incorporated as such. The evidence for these conclusions is as follows.

A bone marrow cell suspension was incubated for 4 hours at 38° under 95 per cent O_2 and 5 per cent CO_2 in a reaction mixture containing 0.2 mg. per ml. of labeled glycine whose specific activity was 58,000 c.p.m. per mg. The proteins were precipitated, washed, and dried and gave 19.3 c.p.m. per mg. On treatment with ninhydrin (with non-radioactive leucine added as a CO_2 carrier) the CO_2 collected as BaCO_3 gave no counts. 237.8 mg. of protein were completely hydrolyzed and the hydrolysate was chromatographed on starch with a mixture of 0.1 N HCl, *n*-propanol, and *n*-butanol, in the proportions of 1:2:1, respectively, as eluting solvent according to the method of Moore and Stein (8). 100 mg. of non-radioactive glycine were added to the hydrolysate before it was chromatographed. The glycine fraction of the eluate was collected and evaporated to dryness, 600 mg. of non-radioactive glycine were added, and the mixture was acetylated by the method of Hebrst and Shemin (9). The crystalline acetylglycine obtained was recrystallized four times. Its specific activity was the same after the fourth recrystallization as after the first crystallization.

A summary of the results and calculations is as follows: protein hydrolyzed, weight 237.8 mg.; c.p.m. per mg. 19.3; total counts 4589 per

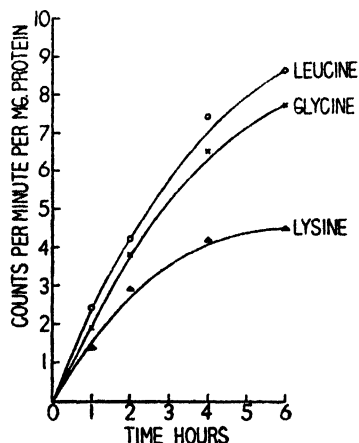


FIG. 1. Rates of incorporation of labeled amino acids by rabbit bone marrow cells. The reaction mixtures contained 0.8 ml. of marrow cell suspension, penicillin G, and CaCl_2 , and the following amounts of amino acids: glycine 0.75 mg., leucine 1.31 mg., and lysine-1- C^{14} 0.047 mg.

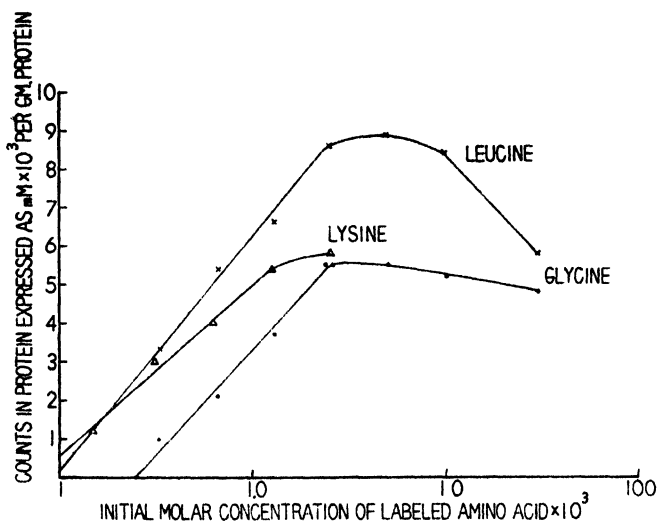


FIG. 2. Effect of initial concentration of labeled amino acid on its rate of incorporation by rabbit bone marrow cells. The results shown are the averages of experiments with and without penicillin G; there were no consistent differences between them, and the differences were within the usual experimental variations. The incubation time was 3 hours, 0.8 ml. of marrow cell suspension was used, and lysine-1- C^{14} was employed. The scale of the abscissa is logarithmic.

minute; glycine added to hydrolysate 700 mg.; glycine in the hydrolyzed protein determined by microbiological analysis 15 mg.; total glycine as

acetylglycine 1115 mg.; c.p.m. per mg. of acetylglycine 1.12; total counts in acetylglycine 1249; per cent of total counts in the protein recovered as acetylglycine $1249/4589 \times 100 = 27$. Therefore 27 per cent of the counts in the protein were due to glycine incorporated as such. Some of the remaining counts were undoubtedly in the heme formed from the labeled glycine (10, 11).

The acetylglycine gave the following elementary analysis.

$C_8H_7NO_2$.	Calculated.	C 41.02, H 6.02, N 11.96
117.10	Found.	" 41.27, " 6.20, " 11.87

The same general method of hydrolysis and chromatography, followed by isolation with non-radioactive carrier, was applied to the proteins obtained after incubating marrow cells with labeled leucine and with labeled lysine. The leucine was isolated as such and accounted for 75 per cent of the counts originally in the protein.

The leucine isolated gave the following analysis.

$C_6H_{11}O_2N$.	Calculated.	C 54.93, H 9.6, N 10.4
131.17	Found.	" 55.00, " 9.76, " 10.5

The lysine was isolated as the monopicrate and accounted for 87 per cent of the counts originally in the protein. The lysine monopicrate isolated gave the following analysis.

$C_{12}H_{17}O_5N_4$.	Calculated.	C 38.36, H 4.57
375.30	Found.	" 39.13, " 4.74

Anaerobiosis and inhibitors of oxidation and phosphorylation inhibited the incorporation of glycine, leucine, and lysine (Table I). The inhibitors used were arsenate, arsenite, azide, and dinitrophenol at 0.001 M concentration in the reaction mixtures. For the anaerobic experiments the reaction vessels were 50 ml. Erlenmeyer flasks fitted with inlet and outlet tubes in rubber stoppers. The flasks were evacuated and then filled with a mixture of 95 per cent N_2 and 5 per cent CO_2 until the pressure was a little above atmospheric; this procedure was repeated six times; the reaction mixtures were then incubated under the N_2 - CO_2 gas mixture.

The results in Table I show that anaerobiosis inhibited completely the incorporation of the labeled amino acids into the proteins. The inhibition was a consequence of the anaerobiosis and not of the evacuation and gassing procedure; this was proved by the finding that the counts given by the proteins were the same when the incubation was carried out under O_2 - CO_2 , whether in beakers or in Erlenmeyer flasks with the repeated evacuation and regassing.

0.001 M arsenite or dinitrophenol was completely inhibitory; arsenate

and azide were nearly so. The relative effectiveness of the four inhibitors was practically the same with the three amino acids.

These observations are in accord with those of other workers. The uptake of methionine into their proteins by non-proliferating *Escherichia coli* is inhibited by azide, fluoride, and cyanide (12). Oxygen consumption and glycine uptake into the proteins of rat liver homogenate go together and the process is inhibited by anaerobiosis (13); anaerobiosis (14) and dinitrophenol (15) inhibit the uptake of alanine into the proteins of rat liver slices.

TABLE I

Effects of Anaerobiosis and of Oxidation and Phosphorylation Inhibitors on Incorporation of C¹⁴-Labeled Glycine, L-Leucine, and L-Lysine into Proteins of Rabbit Bone Marrow Cells

Inhibitor	Labeled amino acid					
	Glycine		L-Leucine		L-Lysine	
	Counts per min. per mg. protein	Inhibition	Counts per min. per mg. protein	Inhibition	Counts per min. per mg. protein	Inhibition
		<i>per cent</i>		<i>per cent</i>		<i>per cent</i>
None.....	3.4		4.2		4.3	
Anaerobiosis ...	0	100	0	100	0	100
Arsenite.....	0	100	0	100	0	100
Dinitrophenol....	0	100	0	100	0	100
Arsenate	0.15	96	1.0	77	0.9	80
Azide.....	0.57	84	1.0	77	1.4	68

The reaction mixtures contained 0.7 ml. of marrow cell suspension. In the experiments with inhibitors 0.1 ml. of Ringer's solution was replaced by 0.1 ml. of one of the following: 0.01 M sodium arsenate (Na_2HAsO_4), arsenious oxide (calculated as As_2O_3), sodium azide, or 2,4-dinitrophenol; the pH in every case was adjusted to 7.0. The amino acids added were glycine 0.2 mg., leucine 0.35 mg., and L-lysine-6-C¹⁴ 0.047 mg.

We have examined the effects of the chlorides of calcium, cobalt, copper, magnesium, and manganese and of potassium phosphate on the incorporation of glycine, leucine, and lysine into the proteins of bone marrow cells. Calcium and magnesium chloride added to a final concentration of 0.004 M were neither stimulatory nor inhibitory.

In the presence of 0.004 M potassium phosphate the uptake of glycine, leucine, and lysine was 77, 67 and 75 per cent of those without added phosphate.

Cobalt, copper, and manganese chloride were inhibitory. Table II gives the averages of results of a number of trials. 0.005 M MnCl_2 appeared to be slightly less inhibitory than 0.001 M.

Evidence has been given above that only about one-quarter of the counts of the protein after incubation with labeled glycine could be accounted for as incorporated glycine. It is noteworthy, in view of this finding, that the different inhibitory agents reduced the total counts in the protein after incubation with glycine to approximately the same

TABLE II

Effects of CoCl_2 , CuCl_2 , and of MnCl_2 on Uptake of Glycine, L-Leucine, and L-Lysine by Proteins of Rabbit Bone Marrow Cells

Molar concentration in reaction mixture of added salt

Uptake as per cent uptake without added metal salt

	glycine	Leucine	Lysine
CoCl_2	100	100	100
0.0001	97	97	100
0.0005	96	65	80
0.001	79	59	60
0.005	58	57	37
CuCl_2	100	100	100
0.0001	71	89	97
0.0005	55	73	65
0.001	46	41	48
0.005	4	7	9
MnCl_2	100	100	100
0.0001	100	95	97
0.0005	85	62	68
0.001	29	13	23
0.005	31	30	35

The reaction mixtures contained 0.7 ml. of marrow cell suspension and 0.1 ml. of the metal salt, 10 times the concentration indicated in the table; when no metal salt was added it was replaced by 0.1 ml. of the Ringer solution; 0.2 mg. of glycine, 0.325 mg. of leucine, and 0.047 mg. of lysine-6- C^{14} .

degree as in the cases of leucine and lysine, in which practically all the counts in the protein could be ascribed to the incorporation of the amino acid as such.

We have found, in the case of guinea pig liver homogenate, evidence that amino acids are incorporated independently of each other (4). The same occurs in marrow cells. Table III gives the result of a typical experiment. The count given by the protein when marrow cells were incubated with three labeled amino acids was the sum of those when each was in the reaction mixture alone. When a reaction mixture contained

one of the three amino acids in the radioactive form and the two others in their non-radioactive forms, the counts were the same as those when only the radioactive amino acid was added to the reaction mixture.

There was no incorporation of labeled amino acids into the proteins when the bone marrow cell suspension was so treated that all the red cells were disrupted. Hemolysis with water or ether, lyophilization, or freezing and thawing six times gave the same result with all three amino acids. With partial hemolysis the amounts of the three amino acids taken up were in proportion to the degree of hemolysis.

TABLE III

Incorporation of C¹⁴-Labeled Glycine, L-Leucine, and L-Lysine into Proteins When Incubated with Rabbit Bone Marrow Cells Separately and Together

Labeled amino acid	Unlabeled amino acid	Counts per min. per mg. of protein
Glycine		1.7
"	Leucine + lysine	1.7
Leucine		2.2
"	Glycine + lysine	2.1
Lysine		4.6
"	Glycine + leucine	3.9
Glycine + leucine + lysine		8.6
Calculated from sum of glycine, leucine, and lysine separately		8.5

The reaction mixtures contained 0.5 ml. of marrow cell suspension. Both the radioactive and non-radioactive amino acids were at the same concentration, expressed as mg. per ml., glycine 2.0, leucine 0.44, and lysine-6-C¹⁴ 0.47.

DISCUSSION

The rates of incorporation of labeled glycine, leucine, and lysine into the proteins of rabbit bone marrow cells from optimum initial concentrations of the amino acids were from the data of Fig. 2, respectively, 0.5, 2.9, and 1.8 $\text{mm} \times 10^{-3}$ per gm. of protein per hour; the value for glycine is on the basis that only 27 per cent of the counts given by the protein came from incorporated glycine. These rates are higher than those that have been observed in tissue slices or homogenates. Rat liver slices formed aspartic acid and glutamic acid from C¹⁴-labeled NaHCO₃, and the sum of the rates of uptake of the two amino acids was 1.4×10^{-3} mm per gm. of protein per hour (16). Expressed on the same basis ($\text{mm} \times 10^{-3}$ per gm. of protein per hour), the rate of incorporation by rat liver slices of alanine was 0.4 (17), of methionine as methionine and cystine 0.003 (18), and of glycine 0.2 to 0.6 (19); the latter values are too high

by 30 (20) to 80 per cent¹ because of adsorbed phosphatidylserine. The rates in the most active fractions of guinea pig liver homogenate (4) were for glycine 0.13, leucine 0.15, and lysine $2.1 \text{ mm} \times 10^{-3}$ per gm. of protein per hour; the value for lysine was that found after 2 hours, at which time the process stops, whereas in bone marrow cells it continues, slowing down only a little during 6 hours.

The rabbit bone marrow cells took up glycine, leucine, and lysine into their proteins as fast as the maximum rates of protein turnover observed *in vivo*, after injecting labeled amino acids (21-23) or after feeding N^{15} -labeled glycine (24).²

In the calculation of the above rates in marrow cells it was assumed that there was no reduction in specific activity of the labeled amino acid in the reaction mixture as a result of dilution by the same but unlabeled amino acid present initially in the cells or formed during the incubation period. The following considerations indicate that it is unlikely that such dilution was large. The volume of the cells was not more than 5 to 8 per cent of that of the whole reaction mixture. The total amino nitrogen concentration in liver is of the order of 0.03 M. As a rough approximation we may assume that the concentration in the marrow cells was the same and that the concentration of any one amino acid is 1/20 of this value, *i.e.* 0.0015 M. In our experiments the optimum initial amino acid concentration was 0.001 M. The reduction of the specific activity of the labeled amino acid by the same amino acid (unlabeled) in the cells was, therefore, of the order of 5 per cent, and the actual turnover rates were, therefore, greater than those given above by about 5 to 8 per cent.

The uptake of labeled amino acids *in vitro* is much faster in embryonic and malignant than in normal adult tissues (17, 25). The data of Zamecnik *et al.* (17) on the uptake of labeled alanine by hepatoma slices give a rate of 1.0×10^{-3} mm per gm. of protein per hour. This rate is slower than that of leucine and lysine and only twice that of glycine in rabbit bone marrow cells. These cells belong, of course, with embryonic tissues.

¹ Personal communication from Dr. D. M. Greenberg.

² Sprinson and Rittenberg's figures give for protein turnover in the whole animal and in the viscera, respectively, 19 and 57×10^{-3} mm of nitrogen per gm. of protein per hour and in man 4.1 and 33×10^{-3} . These figures were obtained after feeding N^{15} -labeled glycine on the assumption that its N^{15} was distributed through all the compounds in the body's labile nitrogen pool and the N^{15} in the proteins was derived predominantly from the nitrogen pool rather than only from the labeled glycine. The validity of this assumption is attested to by the finding that the rate of protein turnover so obtained was nearly the same as that found after feeding deuterium-labeled leucine (24). Sprinson and Rittenberg's figures refer to all the amino acids in the protein. A rough approximation, giving the order of magnitude of rate of average turnover of an individual amino acid, is obtained by dividing the rate of turnover of total protein nitrogen by 20.

Circulating rabbit erythrocytes do not incorporate labeled amino acids into their proteins *in vitro*. The rate in marrow cells appears, therefore, to be associated with their immaturity.

The incorporation of the labeled amino acids into the proteins of bone marrow cells was inhibited by anaerobiosis and inhibitors of oxidation and phosphorylation. This was to be expected if one assumes that amino acid turnover in a protein involves first the rupture and then the reconstitution of peptide bonds (1). But the incorporation of lysine into the proteins of guinea pig liver homogenate is inhibited neither by anaerobiosis, nor, to any marked extent, by the inhibitors of oxidation and phosphorylation, which were effective in bone marrow cells (7). It is premature, therefore, to generalize that the process immediately involved in amino acid turnover in proteins, whether *in vivo* or *in vitro*, is directly coupled with respiration and phosphorylation.

SUMMARY

1. Bone marrow cells incorporate rapidly C^{14} -labeled glycine, L-leucine, and L-lysine into their proteins *in vitro*.

2. After incubating the cells with labeled glycine, leucine, or lysine, 27, 75, and 87 per cent respectively of the counts given by the proteins were accounted for by these amino acids isolated from them.

3. The rate of uptake was, in the case of each of the three labeled amino acids, a logarithmic function of the initial concentration of the amino acid.

4. Anaerobiosis, arsenate, arsenite, azide, dinitrophenol, and disruption of the cells inhibited the uptake of all three labeled amino acids.

5. The chlorides of cobalt, copper, and manganese and potassium phosphate inhibited the uptake of the three labeled amino acids. Calcium chloride and magnesium chloride were neither stimulatory nor inhibitory.

6. Evidence is presented that glycine, leucine, and lysine are incorporated into the proteins independently of each other.

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INCORPORATION IN VITRO OF LABELED AMINO ACIDS INTO RAT DIAPHRAGM PROTEINS*

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We have reported the incorporation of labeled amino acids into the proteins of rabbit bone marrow cells *in vitro* (1), a study that was undertaken to compare the process in intact cells as compared with tissue slices and homogenates. Bone marrow cells are a mixture in different stages of maturity. The uptake of labeled amino acids by rat diaphragm was studied because it is an adult tissue with predominantly one type of cell and it can be removed from the animal with little damage. This preparation has been found useful in studies of carbohydrate metabolism of muscle *in vitro* (2).

The labeled amino acids used were C^{14} -labeled glycine, L-leucine, and L-lysine. The uptake of these amino acids was much slower in rat diaphragm than in bone marrow cells; otherwise the main features of the process were found to be the same in the two tissues.

Procedure

The synthesis of the labeled amino acids used has been described in a previous publication (3). Unless otherwise stated the activities of the amino acids were as follows: glycine-1- C^{14} , 13,400 c.p.m. per mg.; L-leucine-1- C^{14} , 6000 c.p.m. per mg.; L-lysine-1- C^{14} , 15,000 c.p.m. per mg.

The animals were adult white rats in normal nutrition. They were killed by a blow on the head and bled thoroughly. Usually ten to twelve animals were used for an experiment. The diaphragm was removed close to its origin and placed in a small beaker containing cold Krebs-Henseleit Ringer's solution (4). When all the diaphragms were collected, the thick margins, crura, and the central tendons of each were cut away and the remainder was cut along the central axis into two halves. All the halves were collected in a Petri dish containing 20 ml. of ice-cold Ringer's solution; two, taken at random, were then transferred to each reaction vessel.

* This work was carried out under the joint sponsorship of the United States Atomic Energy Commission and the Office of Naval Research. The C^{14} used was supplied by the Carbide and Carbon Chemicals Corporation, Oak Ridge, Tennessee, and was obtained on allocation from the United States Atomic Energy Commission.

The reaction vessels, which were 20 ml. beakers, containing the reaction mixtures were incubated in the apparatus of Dubnoff (5) at 38° under 95 per cent O₂ and 5 per cent CO₂, except in the anaerobic experiments which were under 95 per cent N₂ and 5 per cent CO₂, for 4 hours unless otherwise stated.

At the end of the incubation water was added to each beaker, the pH, in the presence of the tissue, was adjusted to 5.0, the tissue and reaction mixture were homogenized in the apparatus of Potter and Elvehjem (6), and then trichloroacetic acid and water were added to give a volume of 100 ml. of 7 per cent trichloroacetic acid. The suspension stood overnight at room temperature; the precipitated protein was then washed, dried, and its radioactivity measured as previously described (7).

All of the equipment was sterilized before it was used. As a further protection against interference by bacterial action, all of the reaction mixtures contained 1000 units per ml. of penicillin G. We have found in these and other experiments that this concentration of penicillin G keeps the reaction mixtures practically bacteria-free and does not affect the incorporation of labeled amino acids.

The solvent for all the solutions used in making up the reaction mixtures was Krebs-Henseleit Ringer's solution. The volume of the reaction mixtures was 1.0 ml. One component was 0.1 ml. of a solution of penicillin G containing 10,000 units per ml., another the amino acid solution at pH 7.5.

Results

The rate of incorporation into the proteins of glycine, leucine, and lysine proceeded unslackened up to 5 hours (Fig. 1). Zero uptake at zero time indicated on the graph represents experimental values.

Fig. 2 shows that the rate of uptake of each of the three amino acids was a logarithmic function of the initial concentration up to an inflection point; beyond that point there was little or no increase in amino acid taken up with further increase in initial concentration. The inflection point with leucine and glycine was near 0.01 M, with lysine 0.003 M. The values at 0.001 M initial concentration are 0.52, 0.56, and 0.58 mm $\times 10^{-3}$ per gm. (dry weight) of protein for glycine, leucine, and lysine respectively.

Table I summarizes observations on the effects of anaerobiosis and of oxidation and phosphorylation inhibitors. Anaerobiosis, 0.001 M arsenite, or 0.001 M dinitrophenol inhibited the uptake of the three amino acids completely. 0.001 M arsenate and azide were somewhat less inhibitory and the degree of inhibition of the uptake of each of the three amino acids was nearly the same. These findings are very similar to those obtained with rabbit bone marrow cells (1).

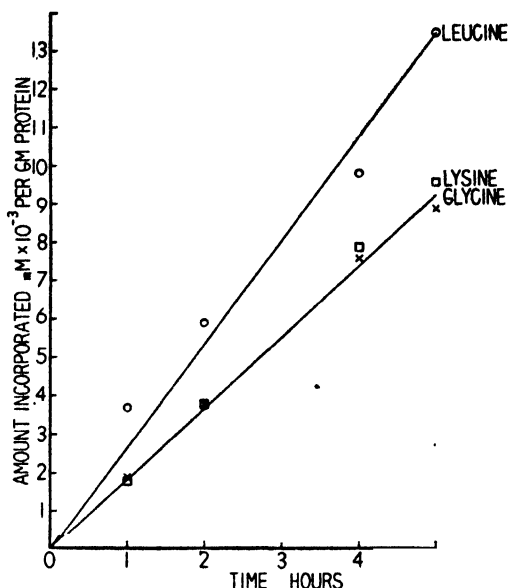


FIG. 1. Rates of incorporation of labeled amino acids by rat diaphragm. The reaction mixtures contained 0.4 mg. of glycine, 0.65 mg. of leucine, and 0.35 mg. of lysine. The zero values at zero time were experimental points.

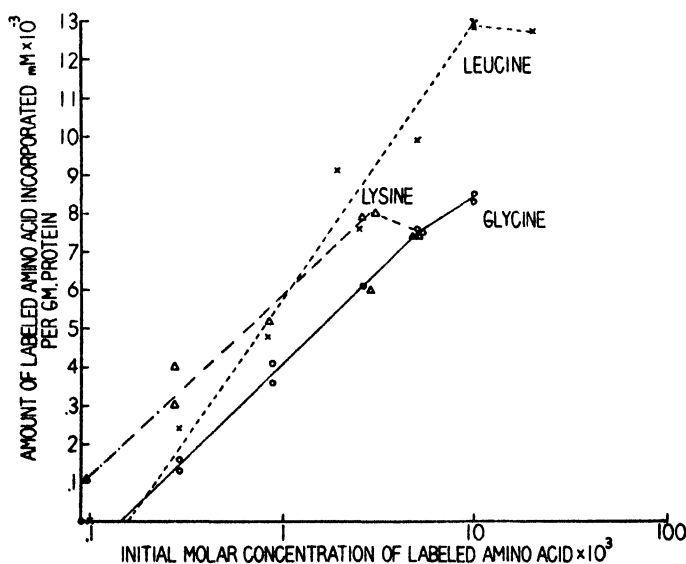


FIG. 2. Effect of initial concentration of labeled amino acid on its rate of incorporation by rat diaphragm. The points on the graphs were from three experiments in which the range of initial concentrations of the three amino acids overlapped. The abscissa scale is logarithmic.

When the tissue was incubated with labeled glycine, leucine, and lysine together, the count subsequently given by the protein was the sum of those when the tissue was incubated with each of these amino acids separately (Table II). This was the same result as that which was found with rabbit bone marrow cells (1) and with a sedimented fraction of guinea pig liver homogenate (3). It indicates that here also the amino acids are taken up independently of each other.

Unlike rabbit bone marrow cells the ability of the diaphragm to incorporate glycine, leucine, and lysine was not completely lost after its cell structure was disrupted by homogenization. The rate of uptake of each of the three amino acids in the homogenized tissue was one-quarter

TABLE I

Effect of Anaerobiosis and of Inhibitors of Oxidation and Phosphorylation on Incorporation of Labeled Glycine, L-Leucine, and L-Lysine into Proteins of Rat Diaphragm

Inhibitor	Glycine		Leucine		Lysine	
	Counts per min. per mg. protein	Per cent inhibition	Counts per min. per mg. protein	Per cent inhibition	Counts per min. per mg. protein	Per cent inhibition
None.....	0.76		0.65		1.63	
Anaerobiosis.....	0	100	0	100	0	100
Arsenite.....	0	100	0	100	0.05	97
Dinitrophenol.....	0	100	0	100	0	100
Azide.....	0.11	85	0.14	78	0.24	85
Arsenate.....	0.25	67	0.27	58	0.60	63

The reaction mixtures contained 0.40 mg. of glycine, 0.74 mg. of leucine, and 0.65 mg. of lysine, and 0.01 ml. of 0.01 M inhibitor, its pH adjusted to 7.5. The inhibitors were sodium arsenate (Na_2HAsO_4), arsenious oxide (calculated as As_2O_3), sodium azide, and 2,4-dinitrophenol.

of that in the intact tissue (Table III). When the diaphragm was boiled prior to its addition to the reaction mixture, there were no counts in the protein at the end of the incubation with either glycine, leucine, or lysine.

Evidence that the labeled amino acids had been incorporated into the proteins was obtained by hydrolyzing them completely with HCl and chromatographing the hydrolysates on starch columns according to the method of Moore and Stein (8). In each case the non-radioactive form of the labeled amino acid with which the tissue had become labeled was added to the hydrolysate before the latter was chromatographed. In the eluates the fractions corresponding to the three labeled amino acids were thus identified by the order in which they emerged and by the increased

color (from the amino acids added to the hydrolysates) they gave with the ninhydrin reagent. And radioactivity was found in the fraction corresponding to the amino acid with which the protein had become labeled.

TABLE II

Incorporation of C¹⁴-Labeled Glycine, L-Leucine, and L-Lysine into Proteins of Rat Diaphragm When Incubated Separately and Together

Labeled amino acid	Unlabeled amino acids	Counts per min. per mg. protein observed
Glycine		0.78
"	Leucine + lysine	0.72
Leucine		0.70
"	Glycine + lysine	0.95
Lysine		2.20
"	Glycine + leucine	2.32
Glycine + leucine + lysine		3.46
Calculated from sum of glycine, leucine, and lysine separately.....		3.68

The reaction mixtures contained 0.4 mg. of glycine, 0.65 mg. of leucine, and 0.74 mg. of lysine.

TABLE III

Incorporation of C¹⁴-Labeled Glycine, L-Leucine, and L-Lysine into the Proteins of Intact, Homogenized, and Boiled Rat Diaphragm

Labeled amino acid	Intact	Homogenized	Boiled
	mm $\times 10^{-3}$ per gm. protein		
Glycine.....	0.83	0.20	0
Leucine.....	1.10	0.28	0
Lysine.....	0.74	0.18	0

The reaction mixtures contained 0.75 mg. of glycine which gave 26,800 c.p.m. per mg., 1.31 mg. of leucine, and 0.37 mg. of lysine. When homogenized diaphragm was used, it was homogenized in the Ringer solution, its pH was adjusted to 8.1, and 0.8 ml. was added to each of the reaction mixtures indicated.

The radioactive amino acids were not isolated and positively identified from the hydrolysates because the uptake of the labeled amino acid by the diaphragm protein was so low. A very large number of animals and of the labeled amino acids would have been required for this purpose, and the cost seemed unwarranted.

DISCUSSION

The incorporation *in vitro* of labeled amino acids into proteins is much slower in diaphragm (rat) than in bone marrow cells (rabbit). For example, at 0.001 M initial concentration, diaphragm took up glycine, leucine, or lysine at a rate of approximately $0.1 \text{ mM} \times 10^{-3}$ per gm. of protein per hour, whereas the rates in rabbit bone marrow cells from the same initial concentration of the amino acids were 0.5, 2.9, and 1.8×10^{-3} mM per gm. of protein per hour respectively.

This difference is in accord with the findings *in vivo*. Thus the data of Greenberg and Winnick (9) give, after intravenous injection of C^{14} -glycine into the rat, after $\frac{1}{4}$ hour rates of 2.6 and 0 $\text{mM} \times 10^{-3}$ per gm. of protein per hour, in bone marrow and muscle respectively, and after 6 hours, hourly rates of 1.2 and 0.88 $\text{mM} \times 10^{-3}$ respectively per gm. of protein.

SUMMARY

1. Rat diaphragm incorporated C^{14} -labeled glycine, L-leucine, and L-lysine into its proteins *in vitro*. The rates were of the order of one-tenth of that in rabbit bone marrow cells *in vitro*. This difference is in accord with the findings *in vivo*.

2. In the case of each of the labeled amino acids the rate of incorporation was a logarithmic function of the initial concentration of the amino acid up to a certain concentration. Beyond that concentration there was little or no further increase in the amount of the amino acid incorporated into the proteins.

3. Anaerobiosis and inhibitors of oxidation and phosphorylation inhibited the uptake of labeled amino acids into the proteins.

4. The rate of uptake of one of the labeled amino acids was unaffected by the presence of the others.

5. The rate of uptake of the amino acids by homogenized diaphragm was one-quarter of that by the intact tissue.

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DOSE-RESPONSE CURVES IN THE ESTIMATION OF POTENCY OF LIPOTROPIC AGENTS*

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In the 18 years which have elapsed since the lipotropic activity of choline was discovered, four other naturally occurring substances have been shown to inhibit the deposition or to hasten the removal of excessive amounts of liver fat and to prevent kidney damage. Dimethylpropiothetin, the one most recently discovered (3, 4), was found in the marine alga *Polysiphonia fastigiata*, but is not known to occur in vegetable or animal products commonly used in human or animal rations. The three other substances (betaine, methionine, and inositol) resemble choline in that they are widely distributed and are present in most natural food products. No *systematic* quantitative study of the relative lipotropic activities of these four common dietary components has been reported.

Numerous exploratory studies of lipotropic phenomena by many investigators have provided a broad general picture of the factors involved. Sufficient data are now available to justify a more systematic attempt to determine the relative activity of naturally occurring and synthetic compounds which affect the accumulation of fat in the liver. Since choline appears to be the effective factor when methionine or betaine is given (5, 6), since it is the most active agent of this type, and since inositol exhibits little, if any, significant lipotropic activity in diets containing a normal amount of fat, it would appear logical to use choline as the standard of reference.

Evidence already in the literature indicates that factors which affect appetite and rate of growth influence the choline requirement. For example, Griffith and his colleagues (7, 8) have repeatedly emphasized that dietary supplements which improve the rate of growth should not be considered antilipotropic since there is no evidence for their direct antagonism to choline but there is proof that an increased demand for choline occurs when growth is promoted. A similar situation with respect to the mineral

* Brief accounts of portions of these studies were presented before the American Society of Biological Chemists at Atlantic City, March, 1946, and at Chicago, May, 1947 (1, 2). The expenses of this investigation were defrayed in part by grants from the Banting Research Foundation.

composition of the diet was noticed by Handler (9). When the minerals were inadequate and growth was very slow, sufficient formation of choline occurred from dietary methionine (of the protein) to maintain normal liver lipides. When the minerals were made adequate, growth was resumed. This appropriated more methionine; less was therefore available for conversion to choline and a fatty liver resulted. Beveridge, Lucas, and O'Grady (10, 11) have shown that the state of a diet with respect to essential amino acids may alter or even reverse the findings in certain types of lipotropic studies. Now that such facts are established, the multiple deficiencies in basal diets containing 5 and 10 per cent of casein (often inadequately supplemented with the vitamin B complex), which we and others have sometimes utilized in the past, must be recognized as factors which may affect seriously the outcome of the experiments and interpretation of the data. That environmental conditions (such as extremes of temperature) may also affect the requirement for lipotropic agents is now established (12).

The choice of a basal diet for comparative lipotropic studies offers unique difficulties. The most awkward one is the fact that the essentiality of methionine as an amino acid precludes its complete removal from the diet. Since elimination of methionine was not considered feasible, a basal diet low in methionine, which has been used in previous studies (11), was adopted for this series of experiments. It does not permit any gain in weight, yet suffices for maintenance of rats which have passed the period of most rapid growth (*i.e.*, initial weight beyond 75 gm.). Although this diet supplies slightly less of several other essential amino acids (threonine, tryptophan, and histidine) than the minimal amounts tentatively proposed by Rose *et al.* (13, 14), it seemed satisfactory for the particular investigation contemplated, *viz.*, a study of the lipotropic relationships in a diet sufficing to maintain weight. In a subsequent paper data will be presented showing the effect of increasing increments of growth upon the choline requirement.

EXPERIMENTAL

In the present study, basal diets have been used containing (Series I) no fat, (Series II) moderate quantities of fat (10 per cent beef fat plus 2 per cent corn oil), and (Series III) a larger amount of fat (30 per cent beef fat). These basal diets (in which the methionine content was deliberately restricted) were, of course, low in organic sulfur. Therefore, in one series of experiments (Series IV) a small supplement of amino acid sulfur (as cystine, 0.4 per cent) was added to the diet containing 12 per cent fat.

Choline, betaine, and inositol have been added in varying amounts to

the basal diets. The lipotropic agents were usually incorporated at levels of 1, 2, 4, 8, 16, and 32 mg. per 10 gm. of food. Choline was supplied as the desiccated chloride in amounts 1.15 times these values and betaine as the hydrochloride in amounts 1.31 times the values. (The effects of methionine supplements will be reported in a separate paper.)

White rats of the Wistar strain (reared in our colony and weighing usually from 70 to 100 gm.) were housed in individual all-metal cages with a false bottom of coarse wire screen. The test diets were fed for 21 days. The groups (containing ten or more animals each) were comparable with respect to weight and sex distribution within each of the four series of experiments. The rats within each series were "group pair-fed" to minimize effects due to differences in food intake of different groups. For example, in Series I, the rats eating the fat-free basal diet tended to consume less food than did those eating this basal diet supplemented with lipotropic substances. Each rat in all of these groups was therefore offered only the average amount of food eaten per day by the group of rats on this basal diet. The care of the animals and the procedures used for the extraction and analysis of liver lipides have been described previously (15).

Series I. Fat-Free Diet—The basal diet for the experiments of Series I possessed the following percentage composition: casein (Smaco, fat-free, vitamin-free) 8, gelatin (Davis) 12, sucrose 72, salt mixture (16) 5, Cellu flour 2, "vitamin powder" 1, cod liver oil concentrate (Ayerst, McKenna and Harrison, Ltd., Montreal; contains 200,000 i.u. of vitamin A and 50,000 i.u. of vitamin D per gm.) 0.015. The "vitamin powder" consisted of thiamine hydrochloride 500 mg., riboflavin 250 mg., pyridoxine hydrochloride 200 mg., calcium pantothenate 1000 mg., and nicotinic acid 1000 mg., made to 1000 gm. with finely powdered (100 mesh) sucrose. This diet supplies 3.7 kilocalories per gm.

Preliminary work had shown that in rats eating such a ration there was a negligible increase in liver lipides during the 1st week, that a rapid rise occurred during the 2nd week, and that during the 3rd week any further increase was questionable (Fig. 1). There appears to be a tendency for the liver lipides to be slightly higher and more variable in female than in male rats. In all subsequent experiments test diets were fed for 21 days (unless otherwise stated) to be sure that the animals were in a condition of equilibrium with respect to liver fat. The weights of rats on this basal diet remained practically constant during this period. Supplements of choline or betaine produced slight gains in weight, while supplements of inositol did not.

The results of these studies may be presented most concisely in graphical form. Fig. 2 shows the amounts of lipotropic supplements added to the test diets, and the resulting total lipide content of the livers. Fig. 3

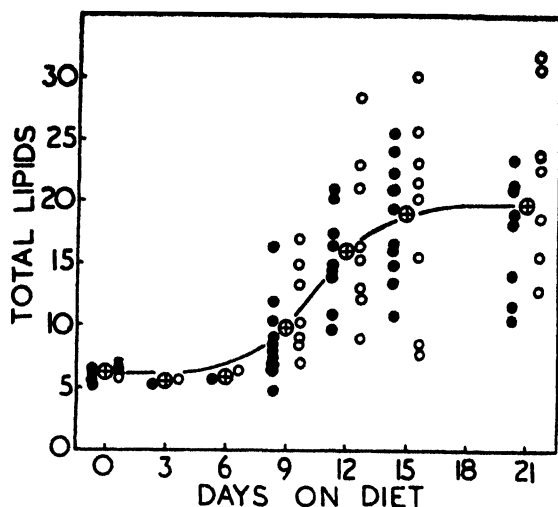


Fig. 1. Rate of development of fatty livers on the fat-free hypolipotropic diet. Total liver lipides expressed as per cent of fresh weight of liver. Individual values for male (●) and female (○) rats (105 ± 15 gm.) are shown with the grand average (⊕). On the 3rd and 6th days the livers were pooled for analysis. Average food consumption about 7.5 gm. per day.

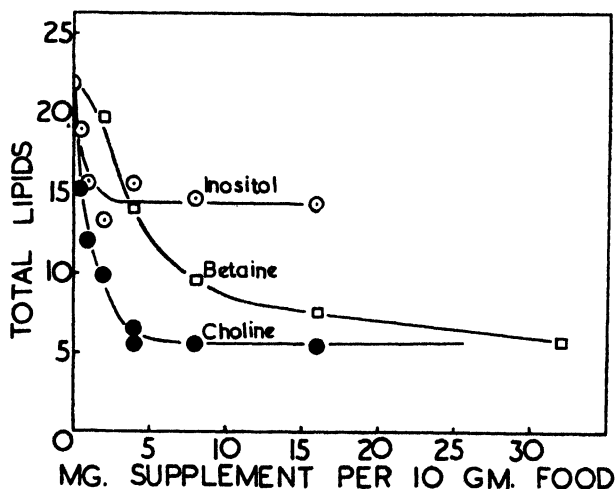


Fig. 2. Dose-response curves showing the effect upon total liver lipides (expressed as per cent of fresh weight of liver) of the lipotropic agents choline (●), betaine (□), and inositol (○) in rats consuming the fat-free diet for 21 days. Ten animals (70 to 90 gm.) per group; average food consumption about 8 gm. daily.

illustrates the supplementary lipotropic effect of inositol when added to diets containing choline or betaine. Fig. 4 shows the effect of choline and

inositol, singly and in combination, on the cholesteryl ester content of the liver lipides.

The amount of each of these lipotropic agents required to produce its maximal effect varied considerably. To maintain the liver lipides at a normal level, about 60 mg. of betaine or 8 mg. of choline per 10 gm. of this type of food were required. In contrast, inositol exerted its maximal effect at very low dosage: 2 to 4 mg. per 10 gm. of food reduced the lipides to approximately 15 per cent, and increasing the dosage up to 32 mg. had no additional effect. The supplementary effects of inositol when added to these fat-free diets containing small amounts of choline are apparently real, as shown in Fig. 3, even if the further decrease in the liver lipides is

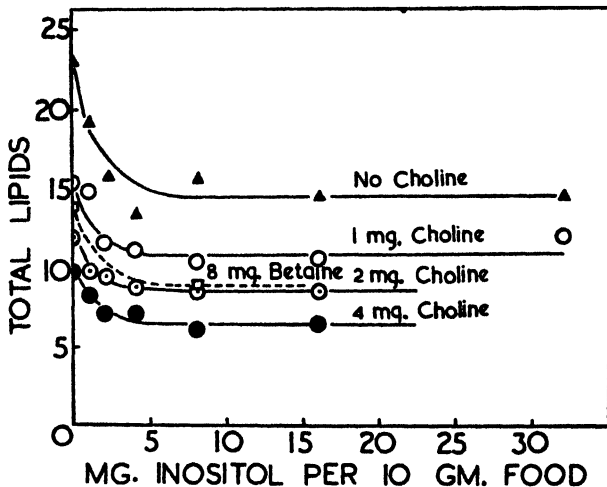


FIG. 3. Dose-response curves illustrating the supplementary lipotropic effects of choline and of inositol in the fat-free diet and of inositol with 8 mg. of betaine per 10 gm. of diet. Total liver lipides expressed as per cent of fresh weight of liver. Rations fed to groups of ten rats each (70 to 90 gm.) for 21 days; average food consumption about 8 gm. daily.

small. Unfortunately these livers were pooled for analysis and hence data are not available for testing the significance of the difference. At maximally effective dosage, choline reduces cholesteryl esters to considerably lower levels than does inositol (Fig. 4), although there is a suggestion that at low dosage levels (below 0.04 per cent in the diet) inositol is somewhat more effective than choline. Inositol exerts a definite but not spectacular supplementary effect upon cholesteryl esters. The ratio of cholesteryl esters to glycerides is not appreciably affected by the presence of inositol in the diets, confirming previous studies (15).

Neither free cholesterol nor phospholipide was altered significantly by the

lipotropic supplements. Since the cholesteryl ester fraction usually accounts for only about 1 to 3 per cent of the total liver lipides, variations in its amount cannot affect the total lipides appreciably. It is obvious, therefore, that choline at all dosage levels exerts its effect mainly upon the glyceride fraction.

Series II. Diets Containing 12 Per Cent Fat—In Series II the diets contained 10 per cent beef fat and 2 per cent corn oil (Mazola) which replaced an equal weight of sucrose in the basal diet used in Series I. The diets of Series II supplied 4.4 kilocalories per gm. The supplements in the test diets and some of the findings are presented in Fig. 5.

Choline is almost as effective in the presence of 12 per cent dietary fat as in the fat-free diet, about 10 mg. per 10 gm. of food giving essentially

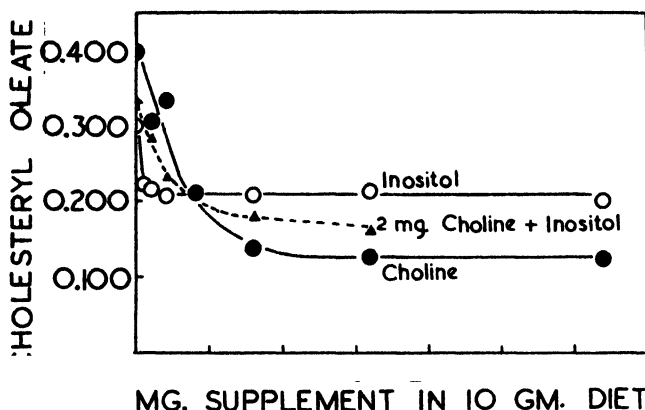


FIG. 4. Effect of lipotropic agents upon cholesteryl esters (expressed as per cent of fresh weight of liver) in liver lipides of rats eating the fat-free diet.

maximal reduction of liver lipides. Owing to the slightly smaller food consumption of these rats, the daily intake of choline required (7 mg.) to produce this effect is essentially the same as in Series I. Inositol failed to exhibit any lipotropic activity whatever in this type of diet.

Series III. Diets Containing 30 Per Cent Fat—These diets, containing 30 per cent of beef fat in place of an equal weight of sucrose in the basal diet of Series I, supplied 5.3 kilocalories per gm. The animals of this series consumed on the average 7 gm. of food per day, corresponding to an average intake of 37 kilocalories as contrasted with 30 and 31 kilocalories in Series I and II, respectively. As might be anticipated, a slightly larger dose of choline was required to give the maximal lipotropic effect with the larger caloric intake. It may be noted (Fig. 5) that the liver lipides did not come down to quite as low a level as they did when less dietary fat was present.

Although betaine was somewhat less effective at lower dosage levels in the diets containing 30 per cent fat than in those free from fat, 64 mg. per 10 gm. of food brought the liver lipides down to normal in both cases. Inositol alone was without effect, and no supplementary action whatever could be detected when it was tested at 1, 2, 4, 8, and 32 mg. levels in diets containing 2 mg. and 8 mg., respectively, of choline.

Series IV. Cystine-Supplemented Diets Containing 12 Per Cent Fat—The basal diet used for Series IV resembled that of Series II but included a supplement of 0.40 per cent cystine. The cystine supplement alone improved the maintenance of weight of the basal group, but little growth

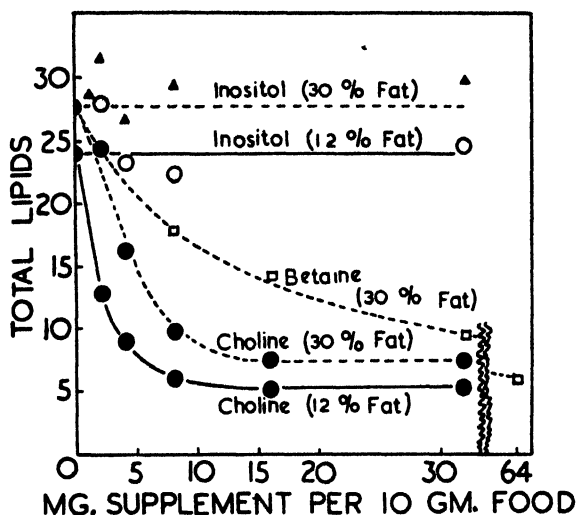


FIG. 5. Dose-response curves showing the total liver lipides (expressed as per cent of fresh weight of liver) resulting from consumption of diets containing 12 and 30 per cent, respectively, of fat with varying amounts of the lipotropic agents. Ten rats (70 to 100 gm.) per group, fed test diets for 21 days; average food consumption about 7 gm. per day.

occurred because other deficiencies (choline first and then possibly threonine and tryptophan) were still limiting factors. The choline supplements that were added to the test diets and some of the findings are shown in Fig. 6. Growth occurred when choline was added, the maximal effect upon weight gain being observed when 8 mg. of choline were present per 10 gm. of food. This increase is possibly due to a sparing influence of choline upon dietary methionine. Choline supplements produced only about one-half as much growth when the cystine supplement was omitted, confirming the suspicion that a deficiency of amino acid sulfur existed in the protein mixture (casein 8 per cent, gelatin 12 per cent of the diet). Inositol

supplements of 2, 4, and 8 mg., respectively, had no beneficial effect upon growth, whether cystine was added or not, and had no effect whatever on the liver lipides (32.6, 34.6, and 32.1 per cent, respectively; basal 33.6 per cent).

The so called "toxic" or "antilipotropic" effect of cystine is seen in the increased deposition of lipides in the livers of the rats on the basal diet. With Griffith (8, 17), we deprecate the application of these terms to cystine, but reserve full discussion of the matter for a future publication. Briefly, the choline requirement is increased by extra growth (which in this case results from an improvement in the organic sulfur content of the ration).

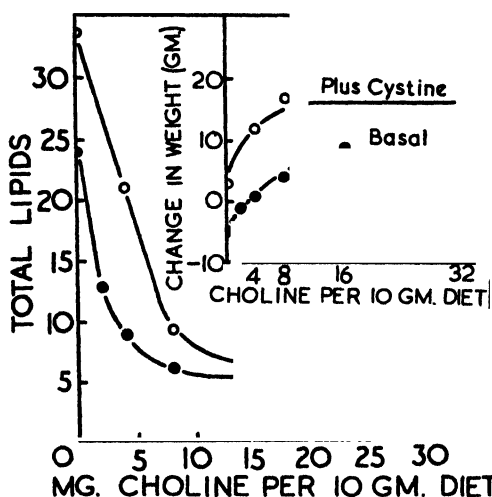


FIG. 6. Lipotropic response of rats (five males and five females per group; 80 to 110 gm.) to different doses of choline in diets with and without supplementary cystine (0.40 per cent). Average food consumption about 7.5 gm. per day. The effects of these supplements on body weight are shown in the insert.

DISCUSSION

Work during the past 25 years on the bioassay of vitamins and on the estimation of the potency of various drugs has shown the necessity of preparing dose-response curves. Extensive investigations of the reliability of such bioassay procedures by chemists, pharmacologists, and statisticians have led to the general recognition of the importance of using only the steep portion of a dose-response curve for making comparisons and have emphasized as another requirement the use of adequate numbers of test organisms. Welch and Welch (18) have already drawn attention to the importance of these considerations in studying lipotropic phenomena. They (19) have reported a dose-response curve for the effect of choline in

mice. In connection with a study of hemorrhagic degeneration of the kidneys in weanling rats, Griffith and Mulford (20) have published a dose-response curve showing the lipotropic effect of choline in young rats on a diet containing 18 per cent casein. No dose-response curves for older rats given any of the lipotropic agents have been found in the literature.¹

It should be appreciated that the naturally occurring lipotropic agents perform functions in the body which are not necessarily associated with their lipotropic activity. All that is ingested may not be used for lipotropic purposes. Indeed, in the case of methionine, data which it is hoped will soon be published suggest that growth requirements tend to be satisfied before labile methyl groups become available for lipotropic action (through choline formation). The total requirement for any one of the natural lipotropic agents probably differs appreciably from the amount used specifically for lipotropic purposes.

Various estimates of the comparative lipotropic potencies of compounds such as choline, betaine, methionine, inositol, "triethylcholine", arsenocholine, etc., have appeared in the literature (18, 20, 21). It has been found that, in weanling rats on a certain diet, choline possesses 3 times the lipotropic potency of betaine and of methionine (20). These observations have been widely accepted as indicating that methionine methyls are efficiently utilized but that probably only one of the betaine methyls is available for lipotropic action. Several years earlier, Platt (21) had suggested that betaine is only about 30 per cent as effective as choline in preventing deposition of fat in the liver. Realization that the nature of the basal diet, the food intake, and the age and sex of the animal (and possibly other factors yet to be determined) all affect the "choline equivalent" of a lipotropic agent makes it very doubtful that a set of conditions happened upon by chance would give ratios of general physiological significance.

It is, therefore, questionable whether such comparative data as are at present available justify the conclusions that have been drawn as to the relative metabolic availability of methyl groups in different compounds.

Even in the simpler case of merely comparing lipotropic potencies, the curves here presented illustrate the grossly erroneous interpretations that may be made if comparisons are based upon arbitrarily chosen doses of these substances. Data presented elsewhere (22) confirm the statement of Griffith (23, 24) that the lipotropic requirement is related to food consumption, *i.e.*, caloric intake. In the present study, since within any one series the average food intakes of rats in different groups are equal, one may compare lipotropic intakes in terms of the amounts in the diets. For

¹ The application of such curves to the comparison of the lipotropic activity of "triethylcholine" with that of choline is reported in a paper from this laboratory (35).

example, if one found (as we report in Fig. 2) in a fat-free diet that a certain amount of choline, say 0.01 per cent, gave the same percentage of liver fat as did 0.32 per cent inositol, the conclusion might be drawn that choline is 32 times as active as inositol; yet when the dose-response curves are available, it may be seen that 0.04 per cent inositol in a diet produces essentially the same amount of liver fat as did 0.32 per cent. Comparison under these conditions leads to the more nearly correct conclusion that choline possesses 4 times the lipotropic potency of inositol. Even this statement of their relative lipotropic potencies is at best only a half-truth, however, since with this dosage of inositol the liver lipides are left at a moderately high level, about 15 per cent, and more inositol does not exert any further effect. In contrast, the addition of more choline to the diet brings the liver lipides down to normal (about 5 to 6 per cent).

This example illustrates the importance of knowing the characteristics of the dose-response curves of the compounds to be compared. It is now well known that unless the dose-response curves of the substances being compared possess essentially similar characteristics, the ratio of potency of one substance with respect to another will vary with the dosage. In other words, a comparison at any *one* dosage level has no general applicability. For example, in Fig. 2 it may be seen that 4 mg. of betaine give the same liver fat value (19.5 per cent) as does about 0.4 mg. of choline. Under these conditions choline is about 10 times as active as betaine; when 8 mg. of betaine are present the liver fat is reduced to 14.3 per cent, a level which is also produced by about 1.4 mg. of choline, giving a potency ratio of just over 5:1. However, if the comparison is made with 32 mg. of betaine (liver fat about 7.5 per cent), the choline requirement for equal reduction of liver lipides is about 5.5 mg. or a potency ratio of 6:1. Finally, since 64 mg. of betaine per 10 gm. of diet are required to reduce the liver fat to normal and only about 8 to 10 mg. of choline are necessary for this purpose, the ratio here is between 8:1 and 6:1. This point has been labored at considerable length in order to show how these ratios are affected by the conditions under which the potencies are compared and that hence no general statement which is applicable to all dosage levels can be made.

It may be mentioned that some years ago, before the ideas discussed above were appreciated, several attempts to compare lipotropic activity were made in which the *decreases* in liver fat of rats on different test diets were compared. However, it is well known that the quantity of fat deposited on a hypolipotropic basal diet may vary considerably, for reasons not yet elucidated. *Mean values* ranging from 20 to 30 per cent of wet liver weight have been encountered when similar (small) groups of rats were fed apparently identical basal diets for the same test period at different seasons. Added choline (say 0.1 to 0.2 per cent) reduces the

liver fat to a fairly constant level (about 5 to 8 per cent). This lower level seems to be much more reproducible than is the high level on the basal diet. Estimates of relative potency, based on decreases measured from the *variable* high level, lack reproducibility because of this variability and are, moreover, meaningless from the point of view of dose-response curves.

Experimental studies and clinical observations during recent years have promoted the lipotropic agents to a prominent position among the dietary components. With an increasing realization of their essential nature, the relative potencies of the various substances possessing lipotropic activity assume greater practical importance. The complex biochemical and physiological interrelationships of these compounds, particularly in the rat and in chicks, have been partially elucidated (25-27). Studies *in vitro* by Borsook and Dubnoff (28, 29) have shown that the enzymes responsible for the various transmethylation reactions are highly specific and that the distribution of the individual enzymes varies from species to species (30).

Raymond and Treadwell (31) have mentioned the desirability of adopting standardized conditions for the comparison of lipotropic activities. Interpretation of the significance of the data they present is impossible because there is no way of knowing whether the one dosage level used in their tests is on the steep part or on the flat portion of the respective dose-response curves. This defect is common to most papers on lipotropic phenomena. Recognition of this weakness is indicated in the recent paper by Welch (27) on the relation of molecular structure of choline analogues and homologues to their protective ability against hemorrhagic renal lesions of dietary origin. Welch reports the effects of eight dosage levels of choline and of two to six levels of the other compounds. There are, of course, commendable features in the proposal to use standardized conditions for comparisons, provided these are chosen with sufficient forethought and with the realization that no one set of conditions can supply a complete answer.

Recent reports (32-34) indicate that vitamin B₁₂ and folic acid exert a sparing action upon the choline requirement. The data presented in this paper show the necessity of having dose-response curves available in order to estimate accurately the requirement for each of the lipotropic agents under different dietary and environmental conditions.

SUMMARY

1. Evidence has been presented showing the necessity of preparing dose-response curves for the accurate estimation of lipotropic requirements under stated dietary and environmental conditions.

2. Dose-response curves are given showing the lipotropic effects of choline, betaine, and inositol in fat-free diets and in diets containing fat.

3. Choline exerts a strong, and betaine a moderate, lipotropic effect under all dietary conditions studied.

4. Inositol exhibits a relatively limited lipotropic effect in fat-free diets but shows no activity when the diet contains fat. The supplementary lipotropic action of inositol (with choline or betaine) in fat-free diets has been confirmed but this supplementary effect is not observed in diets containing fat.

5. The impossibility of making a general statement concerning the activity of any one of the naturally occurring lipotropic substances with respect to any other is obvious from the marked dissimilarity of the dose-response curves.

6. Previously reported lipotropic ratios of 3:1 for betaine and methionine with respect to choline are of questionable significance since the comparisons were made under conditions which have now been shown to be unsatisfactory for establishing such relationships.

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THE MICROBIOLOGICAL DETERMINATION OF PYRIMIDINES WITH LACTOBACILLI*

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It was observed in a preliminary investigation (2) of the pyrimidine requirements of some lactobacilli that *Lactobacillus brevis* 8287 and *Lactobacillus helveticus* 335 appeared to be suitable for the microbiological determination of cytosine, uracil, and thymine. The nutritional requirements of these organisms, the composition of media and conditions near optimal for the assay of the three pyrimidines, and the results of microbiological assays of test mixtures and nucleic acids for cytosine, uracil, and thymine are reported in this paper.

EXPERIMENTAL

The techniques and procedures employed previously in investigating the nutritional requirements and assay potentialities of *Leuconostoc mesenteroides* P-60 (3) have been utilized in the present work. Cultures of *L. brevis* 8287¹ and *L. helveticus* 335¹ were carried as stabs on tomato juice agar (Difco).

Diammonium Uridylate—A sample was isolated from a sulfuric acid hydrolysate of commercial yeast nucleic acid by the method of Levene and Bass (4). The twice recrystallized material was dissolved in hot water, methanol was added, and the crystalline product was dried in air.

Analysis— $C_8H_{11}O_6N_2P(NH_4)_2 \cdot \frac{1}{2}H_2O$. Calculated, N 15.25; found, N 15.35

$[\alpha]_D^{25} = +22.7^\circ$ (1 per cent solution in water). $+22.3^\circ$ was reported by Loring *et al.* (5).

Cytidylic Acid—Nutritional Biochemicals Corporation.

Analysis— $C_8H_{11}O_6N_2P$. Calculated, N 13.00, P 9.56; found, N 12.85, P 9.98

$[\alpha]_D^{25} = +46.9^\circ$ (1 per cent solution in water). $+46.8^\circ$ was reported by Schwerdt and Loring (6).

* Paper 67. For Paper 66, see Camien and Dunn (1). This work was aided by grants from the Nutrition Foundation, Inc., the National Institutes of Health, Swift and Company, and the University of California. The authors are indebted to Dr. M. N. Camien for valuable suggestions. The material in this paper was taken from the thesis of R. B. Merrifield which was presented in June, 1949, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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¹ American Type Culture Collection number.

Cytidine—A solution of cytidylic acid in 10 per cent sulfuric acid was heated for 4 hours at 130°, the hydrolysate was brought to pH 7, and a hot saturated solution of picric acid was added. The crude cytidine picrate, extracted with ether to remove excess picric acid, was recrystallized three times from ethanol. $[\alpha]_D^{25} = +19.3^\circ$ (0.7 per cent solution in 1 per cent HCl). Values of $+19.0^\circ$ and $+20.0^\circ$ have been reported (7).

A solution containing 0.220 gm. of cytidine picrate dissolved in 30 ml. of water was adjusted to pH 4 with H_2SO_4 , extracted with ether, neutralized with $Ba(OH)_2$, filtered, and diluted to 35.0 ml. with water. $[\alpha]_D^{25} = +29.8^\circ$ (0.6 per cent solution in water). The value $+29.6^\circ$ has been reported (4).

Uridine—Cytidine was deaminated to uridine by the procedure of Schwerdt and Loring (6). $[\alpha]_D^{25} = +9.0^\circ$ (0.16 per cent solution in water). The conversion was approximately 83 per cent, calculated on the basis of the value, $+4.8^\circ$, for uridine found by Gulland and Smith (8).

Nucleic Acids—Commercial yeast nucleic acid (Schwarz Laboratories) was purified by the method of Bredereck and Hoepfner (9). The product (vacuum-dried) gave a negative biuret test.

Analysis—Found, N 14.5, P 8.58, N:P = 1.69

A second sample was obtained from the Nutritional Biochemicals Corporation.

Analysis—Found, N 14.0, P 8.50, N:P = 1.65

Sperm desoxyribose nucleic acid (Nutritional Biochemicals Corporation)

Analysis—Found, N 13.5, P 8.58, N:P = 1.57

Purines and Pyrimidines—Commerical samples were recrystallized and analyzed for carbon, hydrogen, and nitrogen. These values were nearly theoretical in all cases.

Nutritional Requirements of L. brevis 8287 and L. helveticus 335

Extensive preliminary experiments were carried out to ascertain optimal levels of amino acids, vitamins, carbohydrates, inorganic salts, buffers, and purines and pyrimidines for use in the basal media. The concentrations given in Table I are the result of this work. The substances which were found essential, stimulatory, or non-essential for the two test organisms are indicated in Table I.

Acid production by *L. brevis* on L-arabinose or L-xylose was nearly twice that on D-glucose or D-fructose (10) but was highest and most rapid when small amounts of glucose were included with the pentose. *L.*

TABLE I
Composition* of Basal Media for *L. helveticus* 335 and *L. brevis* 8237

Constituent	Initial medium	Final medium	
		<i>L. helveticus</i>	<i>L. brevis</i>
	gm.	gm.	gm.
D-Glucose.....	2.00	2.00	0.50
L-Arabinose.....	0	0	3.00
Sodium acetate.....	1.20	1.00	3.00
NH ₄ Cl.....	0.60	0	0
	mg.	mg.	mg.
K ₂ HPO ₄	50.0	100	20.0
KH ₂ PO ₄	50.0	100	20.0
MgSO ₄ ·7H ₂ O.....	20.0	20.0	8.00
FeSO ₄ ·7H ₂ O.....	1.00	1.00	0.40
MnSO ₄ ·4H ₂ O.....	1.00	1.00	0.40
Adenine sulfate.....	2.00	1.00	2.00
Guanine·HCl.....	2.00	1.00	2.00
Hypoxanthine.....	2.00	1.00	2.00
Xanthine.....	2.00	1.00	2.00
Thymine.....	2.00	1.00†	2.00
Cytosine.....	2.00	1.00	0
Uracil.....	2.00	1.00‡	0
Oleic acid.....	0	1.00§	0
DL-Alanine.....	66.7	30.0	61.3
L-Arginine·HCl.....	66.7	30.0	34.8
L-Asparagine.....	66.7	83.3	120.0
L-Cysteine·HCl.....	66.7	30.0	40.0
L-Glutamic acid.....	66.7	100.0	98.8
Glycine.....	66.7	30.0§	17.3
L-Histidine·HCl·H ₂ O.....	66.7	30.0§	17.3
L-Hydroxyproline.....	66.7	0	0
DL-Isoleucine.....	66.7	80.0	64.0
L-Leucine.....	66.7	40.0§	37.3
DL-Lysine·HCl.....	66.7	50.0§	179.0
DL-Methionine.....	66.7	45.0§	24.0
DL-Norleucine.....	66.7	30.0	26.7
DL-Norvaline.....	66.7	30.0	26.7
DL-Phenylalanine.....	66.7	30.0	37.3
L-Proline.....	66.7	30.0	18.7
DL-Serine.....	66.7	120.0	33.3
DL-Threonine.....	66.7	100.0	85.5
DL-Tryptophan.....	66.7	7.5	10.7
L-Tyrosine.....	66.7	15.0§	32.0
DL-Valine.....	66.7	50.0	125.0
	γ	γ	γ
Thiamine·HCl.....	50.0	10.0	8.00
Riboflavin.....	100.0	90.0	6.67
Pyridoxine·HCl.....	80.0	3000	6.67
Pyridoxal·HCl.....	5.00	100	6.67
Pyridoxamine·2HCl.....	5.00	264	6.67

TABLE I—*Concluded*

Constituent	Initial medium	Final medium	
		<i>L. helveticus</i>	<i>L. brevis</i>
	γ	γ	γ
Nicotinic acid.....	100	90.0	600
Calcium DL-pantothenate.....	100	300	60.0
Biotin.....	0.25	0.075	0.10
<i>p</i> -Aminobenzoic acid.....	5.00	10.0	6.67
Choline chloride.....	500	10.0	6.67
<i>D</i> -Inositol.....	1250	10.0	6.67
Folic acid.....	0.050	20.0†	2.00

* Expressed as weight per 100 ml. of final medium.

† Omitted from the thymine assay basal medium.

‡ Omitted from the uracil assay basal medium.

§ Stimulatory.

|| Included in the basal medium although no requirement was demonstrated.

helveticus fermented D-fructose and D-glucose equally well, but did not utilize L-arabinose or L-xylose.

Nucleic Acids and Derivatives—The activities of nucleic acids and derivatives towards *L. brevis* and *L. helveticus* are shown in Table II. *L. helveticus* exhibited a strict requirement for free pyrimidines, while *L. brevis* utilized both free and combined pyrimidines. Uracil, cytosine, orotic acid, uridine, cytidine, diammonium uridyate, and cytidylic acid had the same (or within 10 per cent) activity (on a molar basis) toward *L. brevis*, whereas only uracil and thymine were active toward *L. helveticus*. The slight activity of nucleic acids and guanylic acid for both organisms may be due to adsorbed pyrimidines or to slow enzymatic conversion to active components. All other compounds listed were inactive. It is of interest that orotic acid has been found to be active towards *Lactobacillus casei* (11), group C streptococci (12), and for *pyrimidineless* mutants of *Neurospora crassa* (13). It is utilized by the rat in the synthesis of uracil and cytosine residues of tissue nucleic acids (14).

No gross inhibitions were observed in the present experiments, although acid production was decreased at relatively high (300 γ per tube) concentration of purines and pyrimidines. That purines and pyrimidines may inhibit the growth of microorganisms has been reported by several workers (15–17).

The biosynthetic scheme proposed for *N. crassa* (18) requires that strains which utilize uracil for nucleic acid synthesis must also utilize uridine. If the pattern for lactic acid bacteria is assumed to be similar, the observation that pyrimidine nucleosides are unable to substitute for

the uracil requirement of *L. helveticus* is evidence for a specific function of uracil other than nucleic acid synthesis.

TABLE II
*Uracil and Thymine Activities of Some Pyrimidine Derivatives**

Compound	Mole per cent activity†		
	<i>L. brevis</i>	<i>L. helveticus</i>	
	Uracil assay‡	Uracil assay‡	Thymine assay§
Uracil.....	100.0	100.0	0
Cytosine.....	102.9	0	0
Thymine.....	0	0	100.0
Orotic acid·H ₂ O¶.....	97.2	0.9	0
Uridine.....	91.5	0	0
Cytidine.....	91.2	0.1	0
Diammonium uridylate·½H ₂ O.....	98.7	6.5	0
Cytidylic acid.....	97.4	2.2	0
Guanylic ".....	9.1	3.6	0
Yeast nucleic acid.....	5.7	6.4	1.0
Thymus " ".....	1.2	0.5	2.2
<i>Lactobacillus arabinosus</i> nucleic acid.....	9.4	10.1	0.4
α-Ketoglutaric acid.....	0.6	0	0

* The following compounds had no demonstrable activity toward either organism: adenylic acid, guanosine, uric acid, caffeine, allantoin, alloxan, D-ribose, 2-methoxy-6-aminopyrimidine, 1-D-arabinosyl-5-chlorouracil, and 1-D-arabinosylthymine. The latter two compounds were kindly provided by Dr. Irving Goodman of the University of Colorado.

† The nucleic acid data were calculated on the basis of hypothetical statistical tetranucleotides.

‡ The assay did not detect activities of less than 0.2 per cent.

§ The assay did not detect activities of less than 0.02 per cent.

|| Present in the medium at a high level.

¶ The sample was obtained through the courtesy of Dr. H. K. Mitchell of the California Institute of Technology.

Assay Conditions

Inoculum—Standardized inocula adopted for the assays were prepared by washing 24 hour cultures of *L. brevis* (prepared with the inoculum medium previously described (19)) and diluting the washed suspension to 90 per cent optical transmission. The *L. helveticus* inocula were prepared similarly except that the final washed suspensions were diluted 1:50. The inocula were pipetted with the aid of a Brewer automatic pipette in 0.1 ml. aliquots into 4 inch test-tubes containing final 3 ml. volumes of solution.

Tests showed that growth was slow at 10⁵ or greater dilutions of the

cells washed once (24 hour cultures in 10 ml. of inoculum medium), that the blank values were very high with concentrated inocula, and that the uracil standard curves prepared from data obtained with 18, 24, 48, and 65 hour inoculum cultures were indistinguishable. The uracil assay with *L. helveticus* was not affected by washing the inoculum cells. In the thymine assay with this organism, the blank value was decreased somewhat by washing the cells, owing probably to the removal of folic acid which is active at low concentrations.

Incubation Time—3 days incubation of *L. brevis* at 33° was adopted for the uracil assays, although the data were satisfactory with incubation times of 2 to 7 days. The titration values were about 30 per cent higher at 6 days, compared to 3 days, incubation times, although the useful range of the standard curve was essentially the same and the blank values did not increase appreciably. 3 days incubation of *L. helveticus* at 33° was employed in the uracil assay but, because of the slow acid production in the absence of folic acid, 6 days incubation was used in the thymine assay.

Sterilization Temperature, Sterilization Time, and pH—The most satisfactory conditions found were steaming for 20 minutes at pH 6.8 for the uracil assay with *L. brevis* and steaming for 20 minutes at pH 6.2 and pH 7.4, respectively, for the uracil and thymine assays with *L. helveticus*. Prolonged heating of the medium, especially under pressure, or heating much above pH 6.2 in the uracil assay gave high blank values and low reproducibility of the assay data. The results of prolonged heating under pressure, or of heating the medium below pH 7.4, were similar in the thymine assay. Even at pH 7.4 and 6 days incubation the growth of *L. helveticus* was limited. The analytical ranges found most suitable for the assays were the following (micrograms per 3 ml. tube): uracil or cytosine with *L. brevis*, 1.00 to 15.0; uracil with *L. helveticus*, 0.10 to 1.50; thymine with *L. helveticus*, 0.40 to 3.20.

Recovery of Pyrimidines from Test Mixtures

The test mixtures employed contained all of the constituents of the basal medium in proportions simulating a natural product such as yeast, with the pyrimidine content of the mixture varied from 0.1 to 1.0 per cent. The results of representative recovery experiments are summarized in Table III.

It appears that mixtures containing 0.1 per cent or more of uracil could be assayed accurately with *L. brevis*, but that difficulties might arise in a uracil or thymine assay with *L. helveticus* at levels below 0.5 per cent.

Determination of Pyrimidines in Nucleic Acid Hydrolysates

Hydrolysis of Nucleic Acids and Derivatives—Considerable difficulties have been encountered in hydrolyzing nucleic acids. Purines are liberated

readily by mild acid hydrolysis, but pyrimidine nucleotides are resistant to hydrolysis and problems of incomplete hydrolysis and deamination arise. Literature reports on the hydrolysis of nucleic acids are conflicting (20-24) and indicate the need for further study of the problem.

In experiments on the hydrolysis of nucleic acids and nucleic acid derivatives uracil, cytosine, and thymine were determined microbiologically with *L. brevis* and *L. helveticus*. It was found that uracil was stable under all of the conditions tested (heating at 120° with 3 N, 5 N, and 6 N HCl for 120, 96, and 48 hours, respectively, and at 175° with 90 and 100 per cent formic acid for 2 hours), that the thymine activity was reduced some-

TABLE III
Recovery of Pyrimidines from Test Mixtures

Pyrimidine level*	Per cent recovery (averages of duplicate tubes)								
	Uracil by <i>L. brevis</i>			Uracil by <i>L. helveticus</i>			Thymine by <i>L. helveticus</i>		
	Pyrimidine content of test mixture, per cent								
	1.00	0.50	0.10	1.00	0.50	0.25	1.00	0.50	0.10
1	99	110	100	102	103	121	101	101	104
2	100	101	106	98	107	111	102	99	93
3	98	96	100	111	96	148	98	98	91
4	108	110	116	107	105		102	102	87
5	107	105	97				98	107	84
6							98	97	93
Average....	102	104	104	104	103	127	100	101	92

* The ranges of pyrimidine concentrations (micrograms per 3 ml. tube) employed were as follows: uracil assay by *L. brevis*, 1.00 to 15.0; uracil assay by *L. helveticus*, 0.90 to 3.00; thymine assay by *L. helveticus*, 0.80 to 3.20.

what (10 to 20 per cent) after 6 hours refluxing with 6 N HCl, and that cytosine was deaminated to uracil to the extent of 14 per cent in 96 hours and 23 per cent in 120 hours by heating in 3 N HCl at 120° but only 2.5 per cent in 2 hours by heating in 100 per cent formic acid at 175°.

The uracil liberated from uridylic acid reached a nearly constant value of approximately 90 per cent of theory after 96 hours heating either with 3 N or 5 N HCl at 120°. The 80 per cent recovery of cytosine found by Vischer and Chargaff (23) after heating cytidylic acid 2 hours with formic acid is approximately in agreement with the 86 per cent obtained in the present experiments. The low values reported for uracil in 2 hour formic acid hydrolysates of nucleic acids (23) are in accord with our experience which showed that the activity (measured with *L. brevis*) of uridylic acid, heated for 30 minutes in 90 per cent formic acid, was reduced to 61 per

cent of the initial value. With continued heating the apparent activity increased until, at 360 minutes, the original activity was regained, indicating, therefore, the formation of an inactive or partially active intermediate other than uridine. Uracil plus cytosine, liberated from yeast nucleic acid and sperm nucleic acid, approached a uniform level after 48 to 72 hours heating in HCl, as measured with *L. brevis*. The activity toward *L. helveticus* continued to rise because of cytosine deamination.

The results obtained on a partial HCl hydrolysate of yeast nucleic acid are of interest. The activity of a solution of nucleic acid (at pH 7) toward *L. helveticus* was less than 5 per cent based on the uracil content. It was discovered, subsequently, that contact with 6 N HCl at room temperature produced in 1 minute activity equivalent to 33 per cent of the uracil contained, in 3 minutes to 68 per cent, and in 7 minutes to 1 per cent, while maximum activity was again attained by refluxing in 6 N HCl for 24 hours. These data suggest the liberation and subsequent destruction of an active material, such as a di-, tri- or polyribose nucleotide or possibly a desoxyriboside,² from traces of desoxyribonucleic acid in the sample.

The procedure adopted for nucleic acid hydrolysis consists in heating the sample (1 mg. per ml.) in 5 N HCl at 120° for 48 hours;³ or in formic acid at 175° for 2 hours, in sealed tubes. The hydrolysates are neutralized and diluted to suitable volumes for the assays. All assays are compensated for the sodium chloride or sodium formate present in the hydrolysates.

Pyrimidine Content of Nucleic Acids—Representative data from the present experiments are presented in Table IV. The values for uracil plus cytosine obtained with *L. brevis* at several hydrolysis times were nearly constant for each nucleic acid and are believed to be dependable. The agreement of results from formic acid and HCl hydrolysates supports this view. Since deamination of cytosine is not a factor, the only requisite is that hydrolysis be carried at least to the nucleotide stage without degradation of the pyrimidine ring. The reliability of the uracil values obtained with *L. helveticus* depends upon the degree of deamination of cytosine. Since the uracil value for yeast nucleic acid did not increase

² The desoxyribose nucleotides and nucleosides have not been tested for their activity toward *L. brevis* and *L. helveticus*. However, desoxyribosides are known to be active for several strains of lactic acid bacteria (25-27).

³ This procedure is adequate to destroy folic acid which might be present in the test sample and which would interfere with the thymine assay. 1 mg. samples of crystalline folic acid were heated at 120° with 25 ml. of 0.1 N, 1 N, and 6 N HCl and the hydrolysates assayed for activity with *L. helveticus*. The approximate times required to reduce the activity to 0.1 per cent of the initial value were 24, 12, and 4.5 hours, respectively.

during prolonged hydrolysis, and the value for sperm nucleic acid increased only slightly, the deamination error probably is small.

It may be noted that 1.54 and 1.42 moles of total pyrimidines per 4 moles of phosphorus were found in different samples of yeast nucleic acid. These data, obtained by assay with *L. brevis*, compare well with those (1.54 and 1.48) from assays for total pyrimidine nucleosides made with a *N. crassa* mutant (28), as well as with the values (1.52, 1.39; 1.07)

TABLE IV
Pyrimidine Content of Nucleic Acids

Nucleic acid source	Hydrolysis agent	Time	Pyrimidine content, mole per 4 moles P			
			Uracil and cytosine by <i>L. brevis</i>	Uracil by <i>L. helveticus</i>	Thymine by <i>L. helveticus</i>	Cytosine, calculated
Yeast		<i>hrs.</i>				
	3 N HCl	72	1.56	0.97		
	3 " "	96	1.56			
	3 " "	120	1.53			
	5 " "	48	1.53	0.88		
	5 " "	72	1.52	0.83		
	5 " "	96	1.54	0.91		
Average...			1.54	0.90		0.64
Sperm	3 N HCl	72	0.84			
	3 " "	120	0.84			
	5 " "	48	0.80	0.06		
	5 " "	72	0.79	0.08		
	5 " "	96	0.82	0.09		
	Formic acid	2	0.77	0.03	1.28	
Average....			0.81	0.03*	1.28	0.78

* Since deamination of cytosine is at a minimum in formic acid, only this figure was used.

determined by paper chromatography (24), but they are somewhat higher than the value (1.31) found by chromatographic analysis of total free pyrimidines in hydrolyzed yeast nucleic acid (23).

The molar ratios of uracil to cytosine, 1.41 and 1.22, differ somewhat from the ratios 1.20 and 1.18 (28) obtained by assay with *Neurospora* and the ratios 1.18, 0.78, and 0.95 (24) determined by means of paper chromatography, owing possibly to variations in the composition of the different nucleic acid samples as well as to deamination.

The small increase with hydrolysis time noted for uracil in sperm desoxyribose nucleic acid resulted, it seems likely, from deamination of

cytosine. The minimum value of 0.03 mole of uracil per 4 moles of phosphorus may represent approximately the uracil content of the sample of desoxyribose nucleic acid. The corrected cytosine ratio of 0.78 mole per 4 moles of phosphorus may be compared with the values, 0.88 and 0.54, based on cytosine picrate determined gravimetrically (29, 30). The cytosine ratio for other desoxyribose nucleic acids ranged from 0.52 to 1.02, depending upon the method and the source of these materials. The ratio 1.28 found for thymine in sperm nucleic acid is believed to be reasonably reliable and this value is higher than those (0.89 and 0.92) determined by isolation methods (29, 30). That the molar ratio of thymine to cytosine was 1.64 again illustrates the non-conformity of the experimental data to the tetranucleotide concept of structure for nucleic acids.

SUMMARY

Studies were made of (a) the nutritional requirements of *Lactobacillus brevis* 8287 and *Lactobacillus helveticus* 335 for carbohydrates, vitamins, purines, pyrimidines, oleic acid, inorganic salts, and amino acids, (b) the conditions most suitable for assays of pyrimidines (uracil, cytosine, and thymine), (c) the recovery of these pyrimidines from test mixtures, and (d) the percentages of the three pyrimidines in nucleic acids.

It was found that (a) uracil, cytosine, orotic acid, uridylic acid, and cytidylic acid exhibited approximately equal activity, and that cytidine and uridine showed about 90 per cent of the pyrimidine activity, toward *L. brevis*, (b) none of the compounds tested could satisfy the uracil requirement of *L. helveticus*, (c) the most satisfactory assay conditions were steaming for 20 minutes at pH 6.8 for the uracil and cytosine assays with *L. brevis* and steaming for 20 minutes at pH 6.2 and pH 7.4, respectively, for the uracil and thymine assays with *L. helveticus*, (d) uracil, cytosine, and uracil plus cytosine were recovered satisfactorily with *L. brevis* from test mixtures in concentrations as low as 0.1 per cent and uracil and thymine with *L. helveticus* in concentrations as low as 0.5 per cent, (e) uracil was stable to HCl and formic acid hydrolysis under a variety of conditions, (f) deamination of cytosine to uracil was appreciable during HCl hydrolysis but negligible during formic acid hydrolysis, and (g) liberation of uracil from uridylic acid was only about 90 per cent of theory under the most satisfactory hydrolysis conditions.

The percentages of uracil, cytosine, and thymine in ribose and desoxyribose nucleic acids were determined by the microbiological procedures described and compared to data in the literature.

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VITAMIN B¹² AND GROWTH OF RATS ON DIETS FREE OF METHIONINE AND CHOLINE*

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In studying the rôle of choline in the utilization of homocystine by the rat, du Vigneaud *et al.* (2) observed that an occasional rat would continue growing at a slow rate in spite of the absence of all the "labile methyl" group donors in the diet. This apparent ability of the rat to dispense with intact labile methyl groups in the diet was, however, attributed to the influence of the intestinal bacterial flora. A more thorough investigation by Bennett *et al.* (3) resulted in the observation that the growth of rats deprived of all the known donors of the labile methyl group is not confined to an occasional rat of their colony, but occurs with consistent regularity. In view of the large body of evidence present at that time, indicating inability of the rat to synthesize the labile methyl group, Bennett *et al.* (3) attributed the growth of their rats on diets free from labile methyl to synthetic abilities of the intestinal flora. Apparent confirmation of this opinion was obtained from the results of experiments in which the modification of intestinal flora of the rat was induced by sulfonamide action (4). On addition of vitamins together with rice polishings extract to the sulfonamide diet of the rats, growth was obtained only when the diet also contained methionine. Under these conditions, however, methionine could be replaced by homocystine if additional choline or small amounts of certain liver fractions were added to the diet. Analytical data apparently ruled out methionine, choline, or betaine as effective components. The same authors (4) also observed that, since growth of the sulfonamide-treated rats was not resumed on administration of a purified liver preparation which contained the "active antianemia principle" (now identified with vitamin B₁₂), this factor was also excluded as the possible effective agent in the utilization of homocystine on diets free of the labile methyl group.

Actual synthesis of the methyl groups of choline in the rat was demonstrated by du Vigneaud *et al.* (5), but again this significant observation was attributed to the biosynthetic abilities of the intestinal flora.

* This paper is a part of the report presented before the 116th meeting of the American Chemical Society in Atlantic City, September, 1949 (1). The work was aided in part by a grant from the Williams-Waterman Fund of the Research Corporation, New York.

The capacity of the rat to grow on labile methyl-free diets was not confined to any single strain of rats (4), but was apparently conditioned by the preexperimental diet (6). The restoration of the rat's ability to grow under these conditions by a preliminary feeding of a colony diet was again, however, interpreted as additional evidence "in support of the theory of bacterial synthesis of unknown factors influencing homocystine utilization" (6).

As far as we are aware, at the time of these experiments (2-6) no proof existed that the intestinal bacterial flora of the rat possesses the metabolic activities which result in the production of sufficient amounts of the labile methyl group donors to account for the rates of growth observed or for the significant amounts of choline synthesized in the rat (5). Neither does such evidence exist today.¹ Analysis of rat tissues by the iodometric procedure for methionine, from these rats grown on diets free of the known labile methyl group donors, revealed large amounts of methionine synthesized by the rat (7).

Doubt as to the validity of the interpretation given the data demonstrating the ability of the rat to grow on methionine-choline-free diets (2-4, 6) and to synthesize the methyl groups of choline (5) was further intensified with the appearance of a report by Gillis and Norris (8) that the "animal protein factor" concentrate makes dietary choline unnecessary in animal organism. The availability of crystalline vitamin B₁₂ isolated from liver concentrates and the increasing evidence of its identity with the animal protein factor made possible the testing of vitamin B₁₂ as the factor responsible for the growth of rats on diets free of the labile methyl group. Several years ago, at the conclusion of their studies on the utilization of sulfur-containing compounds by the rat on diets free of labile methyl, Rose and Rice (9) stated that "methionine synthesis from the compounds in question involves the methylation of the sulfhydryl or disulfide group. Whether this reaction is a difficult one for the organism to accomplish, or whether the subnormal growth in our experiments is to be accounted for by an inadequate intake of an active agent, cannot be determined from the data. The findings serve to emphasize the extreme caution with which one must interpret the results of growth studies in which crude vitamin preparations are used." The present communication demonstrates that rats are able to grow on syn-

¹ Upon completion of this work in September, 1949, we attempted to carry out experiments in collaboration with Dr. J. A. Reyniers of the Laboratories of Bacteriology, Lobund, of the University of Notre Dame to test the synthesis of the labile methyl groups in bacteria-free animals. Unfortunately, Dr. Reyniers informed us that the Lobund laboratories were already committed to carry out experiments on an identical problem. We are, therefore, content to await the announcement whether bacteria-free rats are able to synthesize the labile methyl groups.

thetic diets free of methionine and all the known labile methyl group donors when vitamin B₁₂ is added, an observation which has been reported (1).

TABLE I
Composition of Basal Diet

The vitamins and the amino acids were thoroughly ground together before the addition of the other ingredients of the diet.

	gm.		gm.
Glycine	1.0	Lysine·HCl	15.0
Alanine*	4.0	Arginine·HCl	5.0
Serine*	2.0	Histidine·HCl	9.5
Valine*	20.0	NaHCO ₃	13.0
Leucine*	24.0	Sucrose	150.0
Isoleucine*	16.0	Salts (U. S. P. XII)	40.0
Threonine*	14.0	Agar	20.0
Phenylalanine*	12.0	Crisco	100.0
Tyrosine	6.0	Corn oil with 0.5 gm. α -tocoph-	
Tryptophan*	8.0	erol, 65,000 U. S. P. units	
Proline	2.0	vitamin A and 13,000 U. S. P.	
Hydroxyproline	1.0	units vitamin D	20.0
Aspartic acid	4.0	Dextrin	493.5
Glutamic "	20.0		
Total			1000.0

Vitamins per 1000 gm. complete diet

	mg.		mg.
Thiamine·HCl	5	<i>p</i> -Aminobenzoic acid	300
Riboflavin	10	Inositol	1000
Pyridoxine·HCl	5	Folacin	5
Ca pantothenate	5	Biotin	1
Niacinamide	10	2-Methyl-1,4-naphthoquinone	2

* Racemic amino acids.

EXPERIMENTAL

The composition of the basal diet is described in Table I. It is essentially that of Rose and Womack (10), in which methionine, cystine, choline, and liver extract were omitted. All supplements in amounts shown in Tables II and III were mixed with the diet. Crystalline vitamin B₁₂ of Merck and Company, Inc., was used.

Rats of the Wistar strain, born and raised in this laboratory, of either 22 to 24 days or 30 to 32 days of age, were used. A comparison was necessary of the animals of different ages in their ability to survive on the labile methyl-free diets because of the work of Griffith *et al.* (11),

which demonstrated great sensitivity of weanling rats to diets free from labile methyl. Earlier workers, who employed such diets and observed growth of rats, used, as a rule, rats about 100 gm. in weight (3, 4, 6).

All rats were kept in individual metabolism cages with raised screened floors and fed *ad libitum*. Food consumption was recorded. All components of the diet were analytically pure. Homocystine was prepared from methionine, homocysteine from homocystine (12), and a mixture of L-cystathionine and L-allo-cystathionine was synthesized according to a recently published procedure (13). Homocysteine-containing diets were prepared fresh every other day in order to minimize the oxidation of homocysteine to homocystine.

Results

The data summarized in Table II show that 30 to 32 day-old rats continue growing on homocystine-containing diets without vitamin B₁₂ for 2 to 3 weeks, then abruptly lose weight and die with severely hemorrhagic kidneys. If vitamin B₁₂ is administered during the period of loss of weight, the rats immediately resume growth with greatly improved food consumption. All rats continued growing on such diets if vitamin B₁₂ was incorporated at the start of the experiment. The initial growth of rats on vitamin B₁₂-free diets is probably a function of the vitamin B₁₂ which is stored in the liver, and the duration of such growth probably depends on the quantity thus stored. Numerous reports on storage of vitamin B₁₂ in the livers of animals, including rats, have appeared in recent literature. The effects of the preexperimental diet on the ability of the rat to grow on labile methyl-free diets can, therefore, with reasonable certainty be attributed to the storage of vitamin B₁₂ in the liver rather than to "bacterial synthesis of unknown factors influencing homocystine utilization" (6). The importance of vitamin B₁₂ in the diet of mothers, as reflected in the growth, survival, and reproduction of the young, has also been greatly emphasized recently. The variation in the initial growth of rats on labile methyl-free diets obtained in one laboratory (2) as compared to those in another (3, 4, 6) may have been due to differences in the vitamin B₁₂ content of the colony diet and hence to differences in the vitamin B₁₂ content of the livers of the young. Another important variation is in the age of the experimental animals used in the two laboratories. This is illustrated in Table III.

The data in Table II show further that, when a portion of homocystine was replaced by cystine, rats grew better than when homocystine alone was fed. Furthermore, replacing homocystine by homocysteine also resulted in better growth rates. These observations are consistent with our finding (1) that uptake of radioisotopic sulfur of homocysteine into rat tissue cysteine is significantly greater than that of homocystine. Since

apparently the minimal amounts of methionine required for growth were synthesized by the rat from homocystine in the presence of dietary vitamin B₁₂, another limiting factor which prevented the rat from growing better than it did must have been present. This limiting factor appears to be poor synthesis of cysteine from homocystine. Although the rats

TABLE II

Effect of Vitamin B₁₂ on Growth of 30 to 32 Day-Old Rats on Diets Free from Labile Methyl Containing Homocystine, Homocysteine, or Cystathionine

Rat group, male*	Weight		Gain per day	Days on food		Supplements to basal diet
	Initial	Final		Diet	Per day	
	gm.	gm.	gm.		gm.	per cent
1	69	83	1.1	13	7.0	0.8 homocystine
	83	67†	-4.0	4	0.3	0.8 "
2	75	88	1.0	13	6.0	0.8 "
	88	75	-3.2	4	0.5	0.8 "
	75	120	1.3	34	8.0	0.8 " + 15 γ vitamin B ₁₂
3	77	137	1.0	60	7.8	0.8 " + 15 " " "
4	78	102	0.9	27	6.5	0.8 " + 15 " " "
	102	137	2.5	14	9.0	0.5 " 0.3 cystine, 15 γ vitamin B ₁₂
	137	149	0.6	20	7.0	0.8 homocystine, 15 γ vitamin B ₁₂
5	79	148	2.3	30	8.2	0.8 homocysteine, 15 γ vitamin B ₁₂
6	75	74	-0.04	25	6.0	2.0 cystathionine, ‡ 15 γ vitamin B ₁₂
	74	108	1.6	21	7.8	0.8 homocystine, 15 γ vitamin B ₁₂
7	73	68	-0.05	25	6.3	2.0 cystathionine, ‡ 15 γ vitamin B ₁₂
	68	78	0.5	21	6.6	2.0 cystathionine, 15 γ vitamin B ₁₂ , 0.5 choline·HCl

* Each group consisted of six animals. The data on weight gains and food consumption are average values.

† The rats died with hemorrhagic and enlarged kidneys. Rats in Group 2, on autopsy, showed scarred kidneys.

‡ A 50 per cent mixture of L diastereoisomer and L allodiastereoisomer.

grew better on homocysteine, the growth was still suboptimal, indicating that additional limiting factors exist.

A mixture of L-cystathionine and L-allo-cystathionine in lieu of homocystine in the diet failed to stimulate the growth of rats in spite of the presence of vitamin B₁₂ in the diet. On replacing cystathionine with homocystine, growth was promptly resumed. On addition of choline to the cystathionine diet, slow growth was obtained. It has been re-

ported that L-cystathionine is cleaved to cysteine and L-allocystathionine to homocysteine in the rat (14). Our results suggest that under our experimental conditions L-allocystathionine is, perhaps, cleaved to homocysteine, but the extent of such cleavage is small indeed as it is reflected in the growth rate of the rats.

In Table III are summarized data obtained on 22 to 24 day-old rats. Younger animals did not, as a rule, with one observed exception, survive on the labile methyl-free diet in spite of the presence of vitamin B₁₂. Hemorrhagic kidneys, or damaged kidneys in the survivor, were observed in the young rats. Either the synthetic mechanisms of the younger rat

TABLE III

Growth of 22 to 24 Day-Old Rats on Basal Diet Containing Homocystine, Homocystine, or Methionine with or without Additional Choline or Vitamin B₁₂

Rat group, male*	Weight		Gain per day	Days on diet	Food per day	Supplements to basal diet
	Initial	Final				
	gm.	gm.	gm.		gm.	per cent
8	43	53†	1.4	7	4.1	0.8 homocystine, 15 γ vitamin B ₁₂
9	43	49†	0.5	11	4.3	0.8 homocystine, 15 " " "
10	42	36	-1.0	6	1.8	0.7 cystine, 15 γ vitamin B ₁₂
	36	48†	2.4	5	5.0	0.3 " 0.5 homocystine, 15 γ vitamin B ₁₂
11	40	84	1.3	33	7.8	1.4 methionine
12	42	123	2.6	31	8.0	0.8 homocystine, 0.5 choline·HCl

* Each group consisted of six animals. The data on weight gains and food consumption are average values.

† The animals died with hemorrhagic kidneys. One rat of Group 9, however, survived up to the 45th day of the experiment, during which time the animal gained 52 gm. after a loss of 9 gm. 8 days after the beginning of the experiment. The animal was sacrificed on the 45th day and its kidneys showed typical scars

are limited or the requirement for the synthesized metabolites is greater in the younger rat, or both. Another possibility is that the diet, as used by us, is not adequate in certain components which are the precursors for the metabolites that the rat is able to synthesize. When 1.4 per cent of methionine were substituted for homocystine and vitamin B₁₂, the rats grew, but at a suboptimal rate. With homocystine and choline but no vitamin B₁₂ in the diet, the rats grew better than on the methionine diet, but again the growth was not optimal.

DISCUSSION

Our data indicate the probability that the stimulation of growth of rats 30 days or older, which were maintained on homocystine diets free

of labile methyl group donors, by liver preparations (3, 4) was due to vitamin B₁₂ in these preparations. We mentioned in the introduction that the purified active "antianemia principle" preparation, when added to the homocystine diet containing a sulfonamide, failed to stimulate growth (4). This appeared to indicate at the time that the active antianemia principle of the liver, now closely associated with vitamin B₁₂, was not the factor which was responsible for the growth of rats ingesting cruder liver preparations. Bennett (15) has shown recently that, if folic acid is added to the diet containing homocystine and the sulfonamide, growth results for a time and then ceases. On addition to the folic acid-supplemented homocystine-sulfonamide diet of the same preparation of active antianemia principle of the liver which was used earlier (4), growth was resumed. It appears, therefore, that the earlier experiments (3, 4, 6) can be reconciled with our present data on the basis of our thesis that vitamin B₁₂ is the factor responsible for the growth of rats on labile methyl-free diets.

In view of the active protein synthesis in growing animals there appears to be little doubt that methionine must have been synthesized by the organism, since methionine is a component of animal proteins and since none was present in the diet. It must also follow that the methyl group of methionine and of other metabolites containing the methyl group was also synthesized in the rat. The source of the carbon of these methyl groups must have been present in the diet. One such likely source is the amino acids, of which glycine and serine, and other amino acids from which glycine and serine are elaborated, are the most conspicuous possibilities. We are not implying that these amino acids are methyl group donors via metabolic degradation to intact methyl groups, which then participate in what is known as transmethylation. We are suggesting that, in the course of metabolic interconversion of certain amino acids, carbon units are elaborated which serve as the carbon source for the synthesis *de novo* of the methyl groups of certain metabolites, including methionine and choline. Vitamin B₁₂ and other dietary factors may play a decisive rôle in these interconversions, in the synthesis and utilization of the labile methyl group, and possibly in what is known as transmethylation. Subsequent reports will deal with these problems.

SUMMARY

30 day-old or older rats are able to grow on labile methyl-free diets which contain either homocystine or homocysteine and vitamin B₁₂. Younger animals do not survive on such diets and generally develop damaged kidneys. The conclusion is reached that the rat is able to synthesize methionine and, probably, other metabolites containing the

labile methyl group and that vitamin B₁₂ and other dietary factors may play a decisive rôle in the synthesis and the utilization of the synthesized labile methyl groups. The possible rôle of dietary amino acids, particularly glycine and serine in this synthesis of the labile methyl groups *de novo*, is pointed out.

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FORMATION OF FORMALDEHYDE AND FORMATE IN THE BIOOXIDATION OF THE METHYL GROUP*

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When the oxidation of the "biologically labile" methyl group to carbon dioxide was first reported several years ago, it was pointed out that the identity of the intermediates produced in this reaction and their rôles in metabolic processes were matters of considerable interest (1). It seemed probable that these intermediates included single carbon compounds whose normal occurrence and function in higher animals had received comparatively little attention. As an initial approach to this problem, the assumption was made that formaldehyde and formic acid are among the compounds produced in the oxidation of methyl groups found in the body attached to S and N, and experiments were conducted to test the validity of this hypothesis.

In exploratory investigations, it was found that the methyl groups of C¹⁴-labeled methionine, betaine, choline, and sarcosine were oxidized to C¹⁴O₂ by rat liver slices. However, only the methyl group of sarcosine was oxidized to carbon dioxide when liver homogenates were employed. Since homogenates offer advantages over tissue slices with respect to the isolation of metabolic products, sarcosine was used as the source of methyl groups in the subsequent isolation experiments.¹

Evidence of the demethylation of sarcosine to glycine *in vivo* was reported by Abbot and Lewis (3) in 1939. Shortly thereafter, Bloch and Schoenheimer (4) fed N¹⁵-labeled sarcosine to rats and showed by isolation experiments that it was rapidly and directly demethylated to form glycine without deamination. In 1941, Handler, Bernheim, and Klein

* A research grant from the Lederle Laboratories Division of the American Cyanamid Company has made the present investigation possible.

¹ Although sarcosine was prepared in 1847 by Liebig (2) as a degradation product of creatine, no reference could be found in the literature to its occurrence in higher animals. Experiments were therefore undertaken to obtain evidence for the production of sarcosine in the animal body. The experiments have shown that sarcosine is formed in the rat and that the methyl groups of methionine and betaine contribute to its formation (Horner, W. H., and Mackenzie, C. G., *J. Biol. Chem.*, in press). These results indicate that sarcosine may be used to study intermediates formed in the oxidation of methyl groups provided by "biologically labile" methyl compounds.

(5) observed that sarcosine was oxidatively demethylated by liver homogenates. On the basis of the increase in oxygen consumption, the increase in glycine, and a positive color test for formaldehyde, they concluded that 1 mole of sarcosine reacts with 1 atom of oxygen with the production of 1 mole of glycine and 1 mole of formaldehyde.

The present paper is a report of the isolation of formaldehyde and the determination of formate as intermediates in the oxidation of the methyl group of sarcosine by liver homogenates and liver slices, and the extension of these results to the metabolism of the intact animal.

A preliminary report of the isolation of formaldehyde has already appeared (6). At the time that this result was presented at the Detroit meeting of the American Society of Biological Chemists, it was also reported that formate is an oxidation product of the methyl group of sarcosine. Recently Ling and Tung (7) have observed a positive color test for formaldehyde when *N*-methyl-L-amino acids are oxidized by a flavo-protein prepared from liver or kidney. Sarcosine is not oxidized by this enzyme.

Preparations and Methods

Synthesis of Radiosarcosine—Barium carbonate² containing C¹⁴ was converted to radiomethyl iodide by the procedure of Melville, Rachele, and Keller (8). The methyl iodide was condensed with the *p*-toluenesulfonylglycine at 70° to yield *p*-toluenesulfonylsarcosine (9). The latter compound was hydrolyzed in concentrated HCl, and the radiosarcosine was isolated as the *p*-toluenesulfonic acid salt. This salt melted at 186–187°, corrected. There was no depression in the melting point following admixture with an authentic sample.

C₁₀H₁₅O₄NS. Calculated, N 5.36, S 12.26; found, N 5.66, S 12.30

The *p*-toluenesulfonic acid salt of radiosarcosine was converted to sarcosine hydrochloride.³ The melting point was 170–171°, corrected. The radiosarcosine hydrochloride was neutralized with buffer prior to its use in the biological experiments. Hereafter all mention of radiosarcosine will refer to the free base. 1 mg. of radiosarcosine, when oxidized and converted to BaC¹⁴O₃, gave 1.45×10^6 c.p.m., corrected for the background and self-absorption.

Preparation of Homogenates—Non-fasted adult rats of the Rockland

² The radioactive barium carbonate used in this investigation was supplied by the Monsanto Chemical Company, on allocation from the Isotopes Division, United States Atomic Energy Commission.

³ The author wishes to express his appreciation to Dr. Donald B. Melville for his collaboration in the synthesis of radiosarcosine.

Farms strain, that had been maintained on a commercial dog chow ("big red dog food," Co-op Grange League), were stunned by a blow on the head and killed by exsanguination. The liver was removed and homogenized in the Potter-Elvehjem apparatus (10) with sodium phosphate buffer, 0.05 M, pH 7.8, equal in volume to the weight of liver employed. This is the buffer employed by Handler, Bernheim, and Klein (5) in their investigation of sarcosine oxidase. Homogenization and subsequent operations were carried out in a cold room at 7°.

The homogenate was centrifuged for 5 minutes at approximately $2500 \times g$. All, or a portion, of the upper phase, depending on the individual experiment, was then removed and replaced with an equal volume of buffer. The homogenate was mixed and the above process was repeated. The preparation was used immediately.

Warburg Experiments—1 ml. of homogenate and 1 ml. of buffer were added to each vessel. The desired quantity of radiosarcosine dissolved in 0.2 ml. of buffer was placed in a side arm. The incubation was carried out at 37° in an atmosphere of air. In experiments with liver slices 200 mg. of slices in 2 ml. of Krebs-Ringer phosphate buffer, pH 7.4, were placed in each vessel. The incubation was carried out at 37° in an atmosphere of oxygen.

In both the enzyme and liver slice experiments the center well of the Warburg vessel contained 0.2 ml. of 2.5 N sodium hydroxide. Hydrochloric acid was generally used to stop the reaction and to liberate carbon dioxide. Shaking was continued for $\frac{1}{2}$ hour after the addition of acid.

Large Scale Experiments—The incubation was carried out in a 250 ml. filter flask immersed in a water bath at 37°. The ratio between the total volume of buffer and homogenate (or liver slices) was the same as that used in the Warburg experiments. Substrate and reagents were added to the flask by way of a separatory funnel. Carbon dioxide-free air (or oxygen) was drawn through the reaction vessel and thence through a scrubber containing an aqueous solution of dimedon adjusted to pH 4.5 with acetic acid.

Measurement of $C^{14}O_2$ —The sodium hydroxide contained in the center well of each Warburg vessel was transferred to an Erlenmeyer flask and the well was washed out with 5 ml. of water. 21.5 mg. of non-isotopic sodium carbonate were added to the combined solutions, the volume was made up to approximately 25 ml., and the carbon dioxide was precipitated as barium carbonate. The latter compound was deposited on a filter paper disk and counted in a thin mica window Geiger-Müller counter against a barium carbonate standard prepared from the combustion of radiosarcosine. The C^{14} present in the experimental sample was calculated in terms of radiosarcosine, and the percentage of the incubated

— $C^{14}H_2$, oxidized to $C^{14}O_2$, was derived from this value. A detailed account of the preparation of samples for counting will be found in a previous publication (1).

Isolation of $C^{14}H_2O$ —The radioformaldehyde present in the incubation mixture at the end of an experiment was isolated as the dimedon (dimethyldihydroresorcinol) derivative (11), also referred to as formaldemethone. In the homogenate experiments the incubation mixture from each Warburg vessel was transferred, with the aid of 3 ml. of water, to a 15 ml. centrifuge tube. When slices were employed, the contents of each vessel were homogenized in the Potter-Elvehjem apparatus at the end of the incubation period and then transferred to a centrifuge tube. 5 ml. of 20 per cent trichloroacetic acid (TCA) were added and the protein was spun down in the centrifuge. The supernatant was decanted, and the precipitate was washed twice with 5 ml. portions of 10 per cent TCA. The combined TCA solutions were adjusted to pH 4.5 with sodium hydroxide and filtered.

3.7 mg. of non-isotopic formaldehyde were added to the filtrate, followed in a few minutes by 20 ml. of 0.4 per cent aqueous dimedon. After 24 hours the formaldemethone was removed by filtration. Examination of the filtrate showed that this procedure removed 99 per cent of the metabolic $C^{14}H_2O$ present in the TCA-soluble fraction. The isolated formaldehyde was counted directly against the barium carbonate standard prepared from radiosarcosine. The counts were corrected for self-absorption but not for any difference that may exist in the counting rate of formaldemethone and barium carbonate. The dimedon derivative was recrystallized to constant radioactivity. The results were expressed as the per cent of incubated — $C^{14}H_2$, recovered as $C^{14}H_2O$.

Determination of $HC^{14}OOH$ —10 mg. of carrier formic acid and 16 ml. of 0.1 M phosphoric acid were added to the formaldehyde-free TCA-soluble fraction and the solution was distilled into dilute alkali. When the volume in the distilling flask reached 20 ml., 50 ml. of water were added and the distillation was continued. This process was repeated a second time. The recovery of formic acid from solution by this procedure was 95 per cent.

The distillate was adjusted to pH 6 and treated with 20 ml. of the mercuric chloride reagent of Benedict and Harrop (12), which oxidizes formate to carbon dioxide. The reaction mixture was acidified with phosphoric acid and the evolved carbon dioxide was collected in sodium hydroxide solution. The C^{14} content of the carbon dioxide was determined following its conversion to barium carbonate. The results were expressed as the per cent of incubated — $C^{14}H_2$, recovered as $HC^{14}OOH$. The above procedure was used in the routine determination of $HC^{14}OOH$.

In two experiments, radioformate was also obtained from incubation

mixtures of radiosarcosine and homogenized liver by vacuum distillation. The enzymatic reaction was stopped by the addition of 0.6 ml. of 2.5 M phosphoric acid to the main compartment of the Warburg vessel. The contents of the vessel were transferred to a distilling flask, and 115 ml. of water, 3.6 mg. of formaldehyde, and 12.3 mg. of formic acid were added. The mixture was distilled at 22–24° and at a pressure of approximately 15 mm. of Hg. The vapors were collected and frozen in a flask immersed in a cellosolve-dry ice bath.

When 100 to 110 ml. of distillate had been collected, 20 ml. of 0.4 per cent dimedon solution were added to the melted distillate, and the formaldemethone was removed by filtration 24 hours later.

The filtrate, pH 4.5, was aerated with carbon dioxide-free air for 1 hour. At the end of this time 13 mg. of sodium carbonate were added, and the aeration was continued for another hour. As was shown by analysis, this procedure removed any residual $C^{14}O_2$ present in the solution. The presence of $HC^{14}OOH$ was demonstrated by the production of $C^{14}O_2$ when the solution was treated with mercuric ions. The quantity of $HC^{14}OOH$ found by this procedure was approximately 15 per cent less than the quantity found in the routine procedure described above.

EXPERIMENTAL

In the first experiment, in which 460 mg. quantities of radiosarcosine were incubated with a washed liver homogenate, two procedures were employed in isolating $C^{14}H_2O$, as the dimedon derivative, from the contents of the Warburg vessels. In one of these, carrier formaldehyde was added to the TCA-soluble fraction prepared from an incubation mixture, and the formaldehyde was then precipitated as the dimedon derivative. In the second procedure, the contents of the main well of the Warburg vessel were transferred to a flask containing 200 ml. of 0.1 M phosphoric acid, and the mixture was distilled into aqueous dimedon. Carrier formaldehyde was added to the distillate and the formaldemethone that crystallized was removed by filtration.

The formaldemethone prepared by both of these procedures gave over 1000 c.p.m., uncorrected. The material obtained from the TCA-soluble fraction, and twice recrystallized from ethanol-water, possessed an activity of 148 c.p.m. per mg., corrected for background and self-absorption. There was no change in specific activity following a third recrystallization from this solvent pair. The melting point was 188–189°, corrected. The formaldemethone was finally recrystallized from hot isopropyl alcohol. The crystals so obtained gave 148 c.p.m. per mg. When formaldemethone that had been thrice recrystallized from ethanol-water was sublimed at 100° *in vacuo* (1 mm. of Hg), there was no decrease in the specific activity.

In view of the foregoing results, the formaldemethone prepared from

the TCA-soluble fraction and recrystallized three times from ethanol-water was used in the routine determination of $C^{14}H_2O$. The results of triplicate determinations on the same homogenate preparation agreed to within 10 per cent.

Liver Homogenates—The quantity of $C^{14}H_2O$ that was isolated when radiosarcosine was incubated for 1 hour with different liver homogenates is shown in Table I. The experiments are listed in the order of increasing yield of $C^{14}H_2O$. An increase in the accumulation of $C^{14}H_2O$ was accompanied by a decrease in the accumulation of $HC^{14}OOH$ and a decrease

TABLE I
Oxidation of Methyl Group of Radiosarcosine by Liver Homogenates

Experiment No.	Radiosarcosine	Volume removed from centrifuged homogenate*	Oxygen consumption		Oxidation products, as per cent incubated $-C^{14}H_2$			
			Homogenate alone	Increase due to radiosarcosine	$C^{14}H_2O$	$HC^{14}OOH$	$C^{14}O_2$	Total
	μM	per cent	μM	μM				
41	4.77	20	7.2	3.0	2.4	15.1	8.6	26.1
75	3.36	30	6.8	2.1	3.8	14.0	4.6	22.4
71A	5.07	20	6.5	2.7	8.1	12.7	2.6	23.4
11	5.18	60	2.7	2.8	12.9		1.4	
71B	5.07	50†	2.5	2.4	17.4	10.3	0.5	28.2
Controls‡	4.77–5.7	20	0	0	0	0	0	0

* The figures in this column indicate the per cent of the total volume removed from the top of the homogenate and replaced with buffer after the second of two centrifugations. Equal or smaller volumes had been removed after the first centrifugation.

† This homogenate was centrifuged three times; otherwise the general procedure was unchanged.

‡ Homogenates heated for 5 minutes in a boiling water bath.

in the production of $C^{14}O_2$. However, the *sum* of these three oxidation products agreed reasonably well in different experiments.

These results suggested that approximately the same amount of $C^{14}H_2O$ was *produced* by different preparations, but that the quantity that *accumulated* depended on the extent to which formaldehyde oxidase (and formic acid dehydrogenase) had been removed from the sedimented fraction of the homogenate. Evidence for this supposition was found in Experiments 71A and 71B (Table I), in which the homogenates were prepared from the same rat liver, and hence differed only in the extent to which the particulate fraction was freed from the supernatant phase. With the more thoroughly washed homogenate (Experiment 71B), the accumulation of $C^{14}H_2O$ was double that obtained with the less thoroughly

washed homogenate (Experiment 71A). It was observed that the discarded upper phase of the centrifuged homogenates contained formaldehyde oxidase, as indicated by Warburg experiments.

The increase in oxygen consumption that was produced by the addition of sarcosine to a liver homogenate (Table I) was in each instance more than double the calculated quantity required for the production of the observed oxidation products. In Experiment 41, an attempt was made to locate the incubated radiocarbon that was unaccounted for as formaldehyde, formate, and carbon dioxide.

The sodium hydroxide solution obtained from the center well of the Warburg flasks was examined for the presence of $C^{14}H_2O$ and $HC^{14}OOH$. The combined quantity of these two compounds that had been absorbed in the alkali was equivalent to approximately 0.1 per cent of the incubated radiomethyl groups.

The completeness with which $C^{14}H_2O$ had been removed from the TCA-soluble fraction was checked by adding a second quantity of carrier formaldehyde to the filtrate obtained from the first formaldehyde precipitation. This second sample of carrier formaldehyde was reisolated as the dimedon derivative and found to contain less than 0.05 per cent of the C^{14} originally added as radiosarcosine.

The protein fraction (TCA precipitate) was extracted twice with 10 per cent TCA, three times with acetone, and twice with ether. The average C^{14} content of the protein obtained from the experimental vessels was equal to 1.8 per cent of the C^{14} added as radiosarcosine. In contrast, the protein prepared from the boiled homogenate incubated with radiosarcosine contained 0.1 per cent of the added C^{14} .

The radiosarcosine still present in the incubation mixture was determined by the carrier procedure. 50 mg. of carrier sarcosine were added to aliquots of the TCA-soluble fraction obtained from each vessel. The sarcosine was reisolated as the hydrochloride and recrystallized from ethanol-ether to constant specific activity. From the radioactivity determinations, it was calculated that the average experimental vessel contained no more than 1.1 per cent of the original radiosarcosine.

A portion of the acidified TCA-soluble fraction, from which $HC^{14}OOH$ had been removed by distillation, was neutralized and evaporated to dryness *in vacuo* over phosphorus pentoxide. The residue was oxidized by the method of Van Slyke and Folch (13), and the evolved carbon dioxide was absorbed in alkali, converted to barium carbonate, and counted. When corrected for the size of the aliquot employed, the total C^{14} present in the TCA-soluble fraction was found to represent 60 per cent of the incubated radiomethyl carbon, of which 1.1 per cent at the most could be accounted for as radiosarcosine. The remainder was present

as a compound (or compounds) that had been retained in acid solution during distillation.

The total recovery of C^{14} from the experimental vessels, in the form of formaldehyde, formate, carbon dioxide, protein, sarcosine, and one or more unidentified compounds, was 85 per cent. With the boiled homogenate, the total recovery of C^{14} was 92 per cent. 78 per cent was present as radiosarcosine, and the remainder was present as an unidentified compound (or compounds) retained in acid solution during distillation.

The determination of the total C^{14} and the radiosarcosine present in the TCA-soluble material, obtained from both the control and the experimental vessels, was made after the TCA-soluble material had been stored in a refrigerator for approximately 1 month.

Liver Slices— $C^{14}H_2O$ was isolated, as the dimedon derivative, when liver slices were incubated with radiosarcosine. The formaldemethone

TABLE II
Oxidation of Methyl Group of Radiosarcosine by Liver Slices

Radiosarcosine	Oxidation products, as per cent incubated — $C^{14}H_2$		
	$C^{14}H_2O$	$HC^{14}OOH$	$C^{14}O_2$
γ			
300	0.55		4.9
300	0.88	1.9	5.0
300	0.54	1.8	4.8
300*	0	0	0

* Incubated with liver slices heated for 5 minutes in a boiling water bath.

obtained in different experiments gave approximately 75 c.p.m. above the background. No $C^{14}H_2O$ was formed when liver slices were heated in a boiling water bath before the addition of radiosarcosine.

$HC^{14}OOH$ and $C^{14}O_2$ were produced in these experiments, as is shown in Table II. The oxygen uptake of liver slices was not increased significantly by either radiosarcosine or non-isotopic sarcosine.

The conversion of the methyl group of sarcosine to formaldehyde by kidney cortex slices was also demonstrated.

Detection of Free (Volatile) $C^{14}H_2O$ —In the foregoing experiments with liver homogenates and slices, the possibility existed that $C^{14}H_2O$ was not elaborated as such but was formed from a labile precursor during the precipitation of proteins with trichloroacetic acid. The following experiment was carried out to obviate this possibility.

1.03 mg. of radiosarcosine were incubated with a washed liver homogenate in the apparatus described in the section on methods. A slow

stream of carbon dioxide-free air was drawn through the reaction vessel and thence through an aqueous solution of dimedon. After 30 minutes, 3.74 mg. of non-isotopic formaldehyde were added to the reaction mixture. 5 minutes later the flow of air was discontinued, and a rapid stream of nitrogen (approximately 0.5 liter per minute) was drawn through the apparatus for 4 hours. During this time crystals of formaldemethone appeared in the dimedon solution. The scrubber containing dimedon was disconnected and TCA was run into the reaction vessel. $C^{14}H_2O$ was isolated from both the dimedon solution and from the TCA-soluble fraction of the incubation mixture, and the two samples of formaldemethone were recrystallized to constant radioactivity. The results are shown in Table III.

TABLE III
Detection of Free (Volatile) $C^{14}H_2O$ as Oxidation Product of Radiosarcosine

Preparation	Radiosarcosine	Duration of incubation*	$C^{14}H_2O$ as per cent incubated — $C^{14}H_2$	
			Isolated from gas drawn through re- action vessel	Isolated from contents of reaction vessel
	mg.	hrs.		
Sedimented fraction from 1 gm. liver.....	1.03	0.5	3.9	16.6
0.6 gm. liver slices.....	1.51	2.5	0.2	1.2

* At the end of the incubation period a stream of nitrogen was drawn through the homogenate preparation for 4 hours, and a stream of air was drawn through the liver slice preparation for 1 hour. For further details see the text.

A similar experiment was carried out with 600 mg. of liver slices and 1.5 mg. of radiosarcosine incubated in an atmosphere of oxygen. 5.6 mg. of carrier formaldehyde were added to the reaction mixture during a period of $2\frac{1}{2}$ hours. The mixture was then adjusted to pH 2 with hydrochloric acid to stop the reaction and to increase the rate of diffusion of formaldehyde (14), and carbon dioxide-free air was drawn through the apparatus at 1 liter per minute and at a pressure of -70 mm. of Hg. for 1 hour. The quantities of $C^{14}H_2O$ isolated from the dimedon scrubber and from the reaction vessel are given in Table III. The $C^{14}O_2$ produced in the course of this experiment was also determined and found to be equivalent to 5.9 per cent of the incubated — $C^{14}H_2$.

Direct Isolation of Formaldehyde—Because of the importance that is attached to the identification of formaldehyde as a normally occurring compound in the metabolism of animals, it was desirable to exclude com-

pletely the possibility that its isolation with carrier formaldehyde was an artifact attributable to the presence of a radioactive contaminant. Accordingly, an attempt was made to isolate formaldehyde as an oxidation product of the methyl group without the use of a carrier.

The livers from four rats were homogenized in the Potter-Elvehjem apparatus. The homogenate was centrifuged twice, the entire upper phase being removed and replaced with buffer following each centrifugation. The particulate fraction was incubated for 1 hour with 49 mg. of non-isotopic sarcosine plus 1.26 mg. of radiosarcosine. 8 mg. of formaldemethone were isolated from the TCA-soluble fraction of the incubation mixture. The material was recrystallized three times from ethanol-water. Admixture with an authentic sample of formaldemethone did not depress the melting point. The specific activity of the formaldemethone was 730 c.p.m. per mg.

In view of the relatively low yield of formaldehyde (Table IV), and the difficulties encountered in homogenizing large volumes of liver in the Potter-Elvehjem apparatus, the experiment was repeated on a homogenate prepared in the Waring blender. The particulate fraction from 29 gm. of homogenized liver was incubated with 25 mg. of non-isotopic sarcosine for $\frac{1}{2}$ hour. At the end of this time a second 25 mg. portion of sarcosine was added. The reaction was stopped after 1 hour by the addition of TCA. Dimedon was added to the TCA filtrate, whereupon crystals of formaldemethone appeared. After one recrystallization from ethanol-water, the isolated formaldehyde derivative weighed 34.8 mg. The derivative was recrystallized two more times from ethanol-water, and a portion was mixed with an authentic sample of formaldemethone. There was no depression in the melting point.

$C_{17}H_{24}O_4$. Calculated, C 69.86, H 8.22; found, C 69.58, H 8.33

Formaldehyde was not isolated when the sedimented fraction of homogenized liver was incubated alone or in the presence of any of the following compounds: glycine, betaine, monomethylaminoethanol, or choline. However, formaldehyde was formed when 16 mg. of methanol were employed as the substrate. The recrystallized formaldemethone, obtained from the oxidation of methanol by the sedimented fraction of a liver homogenate, melted at 188° , corrected.

$C_{17}H_{24}O_4$. Calculated, C 69.86, H 8.22; found, C 69.60, H 8.59

This experiment was repeated several times and formaldehyde was isolated on each occasion. In control experiments, in which the liver preparation was omitted, methanol was not converted to formaldehyde. The experiments described in this section are summarized in Table IV.

Rate of Oxidation of Methyl Group in Intact Animal—An adult rat,

maintained on a diet containing 1.2 per cent methionine and 0.2 per cent choline chloride (15), was given a single 14.6 mg. dose of radiosarcosine intraperitoneally. At the same time 2 gm. of the basal diet were administered by stomach tube. The animal was then placed in a metabolism

TABLE IV
Direct Isolation of Formaldehyde without Use of Carrier Formaldehyde

Weight of liver before homo- genization	Substrate	Formaldem- thone isolated	Yield of CH_2O from $-\text{CH}_3$
gm.		mg.	per cent
30*	50 mg. radiosarcosine	8.0	5
29	50 " sarcosine	34.8	21
53	None added	0	
31	172 mg. glycine	0	
33	92 mg. choline chloride	0	
31	174 mg. betaine hydrochloride	0	
27	18 mg. methanol	9.8	6
31	36 " "	10.1	3

* Homogenized in the Potter-Elvehjem apparatus. In subsequent experiments the homogenate was prepared in the Waring blender.

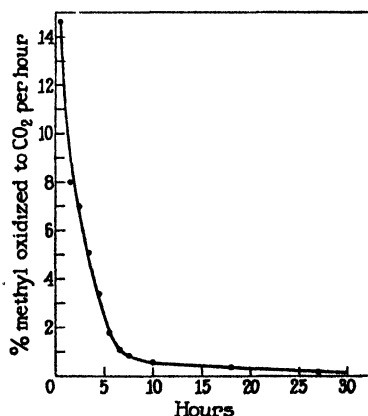


FIG. 1. The rate of oxidation to CO_2 of the methyl group of sarcosine. A single 14.6 mg. dose of sarcosine, labeled with C^{14} in the methyl group, was injected intraperitoneally into a 160 gm. male rat, and the C^{14}O_2 eliminated in the expired air during the next 30 hours was determined.

apparatus (1) and the quantity of C^{14}O_2 that appeared in the expired air during the next 30 hours was measured.

The maximum rate of oxidation of the methyl group to carbon dioxide occurred during the 1st hour (Fig. 1). At the end of 6 hours, 40 per cent, and at the end of 24 hours, 50 per cent of the injected radiocarbon had

appeared in the expired air as $C^{14}O_2$. 4.8 per cent of the administered methyl carbon was excreted in the urine, and 0.35 per cent was eliminated in the feces, in 24 hours. Thus, 55 per cent of the administered methyl carbon was eliminated in 24 hours, and the remainder was still present in the body in the form of methyl groups and their oxidation products.

It should be noted that the oxidation curve for radiosarcosine *reflects* not only the rate of formation of $C^{14}O_2$ but also the rate of formation of the intermediates produced in the oxidation of the methyl carbon.

*Conversion of Methyl Groups to Formate in Intact Animal*⁴—Rats weighing approximately 200 gm. were placed on a purified diet (Table V) and transferred to metabolism cages. The urine was collected daily in

TABLE V

*Excretion of Formic Acid by Rats Following Intraperitoneal Injection of Sarcosine, Glycine, and Methanol**

Weight of rat	Compound injected	Quantity injected daily	Formic acid excreted in urine during injection period	
			1st day	2nd day
gm.		gm.	mg.	mg.
237	Sarcosine	1	17	24
223	"	1	6	
248	"	1	9	13
218	Methanol	0.8	2	7
234	"	0.8	5	24
190	Glycine	1	0.4	0
159	"	1	0.1	1.3

* The percentage composition of the experimental diet was as follows: sucrose 55.4, casein 20.0, Covo 19.0, corn oil 1.0, salts 4.0 (15), L-cystine 0.4, choline chloride 0.2, and vitamins (15).

15 ml. of 0.1 N sodium hydroxide. The average daily excretion of formate, as determined by the method of Benedict and Harrop (12), was 1 (0.2 to 2.0) mg.

The intraperitoneal injection, twice daily, of 0.5 gm. of sarcosine, dissolved in 1 ml. of warm water, was followed by a prompt and definite increase in formate excretion (Table V). The administration of methyl groups in the form of methanol also increased the urinary excretion of formate. The injection of glycine on the other hand produced no discernible increase in urinary formate.

Direct evidence that the methyl group of sarcosine is converted to formate was obtained by adding 3.58 mg. of radiosarcosine to the non-

⁴ The author wishes to express his appreciation to Miss Rose Lubschez for her collaboration in the experiments reported in this section.

isotopic sarcosine administered on the 3rd day of an injection experiment. The urine collected in the ensuing 24 hours was acidified and distilled into dilute alkali. The alkaline solution was then distilled into aqueous dimedon. When three-quarters of the original volume had been distilled, the solution that remained in the distilling flask was adjusted to pH 4 and aerated for 2 hours with carbon dioxide-free air. At the end of this time the solution was shown by analysis to be devoid of $C^{14}O_2$. The solution was then adjusted to pH 6 and oxidized with mercuric chloride reagent. The mixture was acidified and the carbon dioxide, that had been formed in the oxidation, was trapped in dilute alkali and converted to barium carbonate. The activity of the barium carbonate was 253 c.p.m., corrected. It was calculated from this figure that 0.3 per cent of the injected radiomethyl groups had been excreted as $HC^{14}OOH$.

The distillate that had been collected in aqueous dimedon (see above) was treated with carrier formaldehyde and the formaldemethone was removed by filtration. The formaldemethone was counted until 1700 counts had been obtained on four different occasions. The average count above the background was 6.5 (6.0 to 7.3) c.p.m., uncorrected. This level of activity was equivalent to 0.6 γ of the injected radiosarcosine. The formaldemethone was sublimed *in vacuo* (1 mm. of Hg) at 100° without a loss in specific activity. In a control experiment in which 0.75 mg. of radiosarcosine was added to rat urine and allowed to stand for 24 hours, the formaldemethone obtained from the urine by the procedure outlined above contained no detectable C^{14} . $HC^{14}OOH$ was also absent (<0.002 per cent).

The excretion of radioformate following the administration of 3.3 mg. of radiobetaine (16) was also demonstrated. The betaine was added to the non-isotopic sarcosine injected on the 3rd day of a metabolism experiment. 0.8 per cent of the administered radiomethyl carbon appeared in the urine as $HC^{14}OOH$ in 24 hours. Prior to the determination of formate any $C^{14}H_2O$ present in the urine was removed as the dimedon derivative. cursory examination of the formaldemethone indicated that it possessed several counts per minute above background. When 3.5 mg. of radiobetaine were added to acidified urine in a control experiment, and the mixture was distilled, a small amount of $C^{14}H_2O$ was formed. The $C^{14}H_2O$ present in the distillate was equivalent to 0.02 per cent of the added radiobetaine. The radioformate present in the distillate was equivalent to 0.002 per cent of the added betaine.

DISCUSSION

In the experiments described in this paper radioformaldehyde was repeatedly isolated as the dimedon derivative from liver homogenates and

surviving liver slices incubated with radiosarcosine. Radioformate was determined in these preparations by the classical oxidation-reduction reaction between formate and mercuric ions. Methyl alcohol was found to resist oxidation by the mercuric chloride reagent, and other investigators have shown that oxalate, acetate, lactate, pyruvate, etc., are not oxidized by mercuric ions (17, 18). Accordingly, the oxidation of the methyl group of sarcosine may be *schematically* represented as follows:



where X represents the initial product (or products) formed in the reaction. It is quite possible that both formaldehyde and formate are hydrated prior to dehydrogenation and that bicarbonate ion is thus the end-product of the oxidation. With respect to the compound (or compounds) produced in the conversion of the methyl group of sarcosine to formaldehyde and formate, two observations are of interest. First, it was shown, in experiments of a purely qualitative nature, that the sedimented fraction of liver homogenates oxidizes both sarcosine and methanol to formaldehyde. Secondly, the incubation of radiosarcosine with a liver homogenate for 1 hour gave rise to a product that was soluble in trichloroacetic acid and accounted for a major fraction of the added C^{14} . This compound (or compounds) was not removed from acid solution by distillation. Both of these findings are the subjects of further investigations. Ratner, Nocito, and Green (19) have shown that sarcosine is converted to methylamine by glycine oxidase. With respect to methyl alcohol, it is of interest that recent investigations by du Vigneaud and Verly (20) have shown that the administration of $C^{14}H_3OH$ to the rat results in the appearance of C^{14} in the methyl groups of choline and creatine.

The significance of the series of reactions depicted above is not confined to sarcosine. Collateral experiments have shown that the methyl groups of methionine and betaine contribute to sarcosine formation in the body. It follows from this observation that at least part of the methyl groups provided by these compounds are oxidized by way of the same series of reactions. The same is true for methyl compounds (such as choline and dimethylthetin) that contribute methyl groups, directly or indirectly, to methionine and betaine. Thus all "biologically labile" methyl groups are sources of formaldehyde and formate in the body. When radiobetaine was administered to a rat with formaturia, $HC^{14}OOH$ was excreted in the urine.

The results presented in this paper appear to provide the first direct evidence that formaldehyde is a normally occurring compound in the animal body. While nothing is known as yet concerning the fate of formaldehyde in the body, other than its oxidation to formate, it is highly

probable that such a reactive molecule will be found to play an important rôle in synthetic reactions, particularly in reactions involving proteins and amino acids.

Formic acid, on the other hand, has long been known to occur in small amounts in the urine of man and other animals, although information concerning the identity of its precursors, or the extent of its formation in the body, has been scanty. Dakin, Janney, and Wakeman (17) studied the effect of diet on formate excretion, and reached the conclusion that it was produced in the breakdown of carbohydrates and proteins. When Shemin (21) found that serine was converted directly to glycine in the body, he suggested that formate was a product of this reaction. Recently, Siekevitz, Winnick, and Greenberg (22) and Siekevitz and Greenberg (23) reported the presence of HC^{14}OOH in liver slices incubated with glycine labeled in the α -carbon. HC^{14}OOH was identified by oxidation to carbon dioxide with mercuric ions.

The identification of both methyl groups and glycine as sources of formate in the animal body is of considerable interest in view of the papers that have appeared in the past few years on the participation of exogenous formate in purine and serine syntheses. The first of these papers was the report by Buchanan and Sonne (24) and Sonne, Buchanan, and Delluva (25) that the carbon of exogenous formate was incorporated into positions 2 and 8 of uric acid by the pigeon. This was confirmed by Karlsson and Barker (26), who showed that the α -carbon of glycine also entered these positions. Greenberg (27) found that the addition of radioformate to pigeon liver homogenates resulted in the synthesis of radiohypoxanthine, and Marsh (28) observed that the administration of radioformate to pigeons resulted in the appearance of C^{14} in the adenine and guanine of the nucleic acids. However, in similar experiments with rats, Marsh (28) found that all of the C^{14} that entered into purines could be accounted for by C^{14}O_2 fixation.⁵

Evidence that glycine is converted to serine by rat liver homogenates was obtained by Winnick, Moring-Claesson, and Greenberg (29), although

⁵ In an experiment conducted in this laboratory in collaboration with Dr. William H. Horner a rat was injected with 24 mg. of L-methionine, labeled with C^{14} in the methyl group, and the animal was sacrificed 30 hours later. Adenine was isolated from the visceral nucleic acids. The specific activity of the adenine carbon was double the specific activity of the respiratory carbon. Consequently, the specific activity of the *newly synthesized* adenine must have been considerably greater than that of the expired carbon dioxide. It may be concluded that the methyl carbon of methionine contributed to adenine synthesis, probably by way of formaldehyde or formate. In a counter-current distribution of the isolated adenine, performed through the kindness of Dr. George B. Brown, the distribution of C^{14} paralleled the distribution of the adenine, thus indicating that the C^{14} was a part of the adenine molecule and not present as a contaminant.

previous experiments by Greenberg and Winnick (30) had failed to demonstrate this reaction in the whole animal. Sakami (31) showed that glycine, labeled in the carboxyl group, was converted to serine in the intact animal, and that the carbon of exogenous formate appeared in the β -carbon of serine. Subsequently, Goldsworthy, Winnick, and Greenberg (32) also were able to demonstrate the synthesis of serine from glycine in the intact animal.

The condensation of the α -carbon of 1 molecule of glycine with a 2nd molecule of glycine to yield serine was reported independently by Siekevitz, Winnick, and Greenberg (22, 23), on the basis of experiments with liver slices, and by Sakami (33), on the basis of experiments in the whole animal. Sakami (34) has recently shown that the methyl carbon of choline is also found in the β -carbon of serine, a finding that is in accord with the observation that methyl groups are converted to formaldehyde and formate. Wittenberg and Shemin (35) have recently reported that the α -carbon of glycine enters the methine bridge of heme, apparently as a single carbon molecule.*

The experiments cited above suggest that formate, or a derivative of formate, participates directly in the synthesis of purines, porphyrins, and serine. On the other hand, it is possible that the single carbon compound involved in these reactions is formaldehyde, or a radical or compound derived from formaldehyde. In either case, it seems probable that our present knowledge of single carbon compounds provides us with but a glimmering of the rôle they eventually will be found to play in synthetic reactions in the animal body.

Plaut, Bethel, and Lardy (37) have reported that folic acid functions in the metabolism of formate, and evidence is rapidly accumulating that vitamin B₁₂ is concerned with the metabolism of methyl groups (38-41).

The author wishes to express his appreciation to Dr. Vincent du Vigneaud for his advice and counsel during the course of this investigation.

* In an experiment performed in this laboratory several years ago, hemin crystals were isolated from the blood of a rat that had received 52 hours earlier a single 200 mg. dose of L-methionine, labeled with C¹⁴ in the methyl group. The activity of the administered methionine was 1.12×10^6 c.p.m. per mg. The carbon dioxide expired throughout the period of the experiment showed an average activity of 244 c.p.m. per mg. of carbon. The carbon of the hemin possessed 161 c.p.m. per mg. Presumably about 2 per cent of the total heme in the blood was synthesized in the course of the experiment (36). On the basis of this figure, the activity of the newly formed heme was approximately 8000 c.p.m. per mg. of carbon. If it is assumed, in the light of the results of Wittenberg and Shemin (35), that most of this activity was in the methine carbons, it may be calculated that approximately 4.6 per cent of these carbon atoms were furnished by the administered methyl carbon.

Appreciation is expressed to Mrs. Josephine T. Marshall for the micro-analyses, and to Mr. Harry L. Isrow for technical assistance in all phases of the work.

SUMMARY

The experiments reported in this paper indicate that formaldehyde is a naturally occurring compound in the animal organism. $C^{14}H_2O$ was isolated, as the dimedon derivative, from incubation mixtures composed of radiosarcosine and either liver homogenates or surviving liver slices. Free formaldehyde was formed in these preparations, as was shown by blowing $C^{14}H_2O$ directly out of incubation mixtures with a stream of gas. $C^{14}H_2O$ was isolated from the sedimented fraction of liver homogenates both with and *without* the use of non-isotopic carrier formaldehyde.

$HC^{14}OOH$ and $C^{14}O_2$ were also produced in the oxidation of radiosarcosine by liver homogenates and liver slices. The results obtained with different homogenate preparations were consonant with the view that most or all of the formate and carbon dioxide formed in the oxidation of the methyl group arose by way of formaldehyde.

In the whole animal, the administration of radiosarcosine was followed by the elimination of $HC^{14}OOH$ in the urine. A trace of $C^{14}H_2O$ was isolated from the urine as the dimedon derivative. The methyl groups of sarcosine were oxidized at a rapid rate in the body, as was shown by following the $C^{14}O_2$ eliminated in the expired air.

Since collateral experiments have shown that the methyl groups of methionine and betaine contribute to sarcosine formation in the rat, it follows that "biologically labile" methyl groups as a class are sources of formaldehyde and formate in the body. When radiobetaine was administered to a rat with formaturia, $HC^{14}OOH$ appeared in the urine.

The significance of these results with respect to the rôle of single carbon compounds in biosynthetic reactions is discussed, and a brief account is given of experiments which indicate that the carbon of methyl groups contributes to the synthesis of the adenine of nucleic acids and the porphyrin of hemoglobin.

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ON THE METABOLISM OF β -ALANINE*

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Preparatory to a study of the metabolism of anserine and carnosine, β -alanine, synthesized with isotopic nitrogen (N^{15}), was injected into fasting rats for the purpose of measuring the extent of its retention. The results of this experiment seemed of sufficient interest to warrant a brief description at this time, for it was found that under the conditions employed β -alanine is extensively degraded. Estimates made of the rate of urea formation indicate that β -alanine is more rapidly deaminated than either L-aspartic acid or glycine, whereas the rate of incorporation of the nitrogen of the three amino acids into peptide bonds is the same.

EXPERIMENTAL

N^{15} -Labeled β -alanine was synthesized from isotopic phthalimide and methyl acrylate by a method similar to that described by Rodionov and Yartseva (1). N found, 15.8 per cent;¹ theory, 15.9 per cent; N^{15} , 33.1 atom per cent excess.

Animal Experiment—After fasting for 24 hours, five male Sprague-Dawley rats, weighing 320 to 360 gm., were injected intraperitoneally with a 0.85 per cent sodium chloride solution of β -alanine. 1 ml. of this solution, containing 1.484 mg. of nitrogen (0.492 mg. of N^{15}), was administered per 100 gm. of body weight. The animals were placed separately in metabolism cages, and urine was collected for 10 and 48 hours after injection. In order to secure adequate volumes of urine, 0.85 per cent sodium chloride solution (1 ml. per 100 gm. of body weight) was injected intraperitoneally at 2 hour intervals during the first 10 hours of each experimental day. At the termination of each period of collection, cages and funnels were rinsed, and the urine and washings made to convenient volumes. After filtration, aliquots were removed for quantitative and isotopic analyses of the total nitrogen (micro-Kjeldahl), and, where indicated, for urea (precipitation with xanthidrol) and ammonia (adsorption on permutit followed by distillation).

* This work was supported in part by grants from the American Cancer Society, recommended by the Committee on Growth of the National Research Council, and from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

¹ Corrected for N^{15} content.

TABLE I
Excretion of N^{15} in Urine after Administration of N^{15} -Labeled β -Alanine to Fasting Rats

Rat No.	N^{15} given	Total N, 10 hrs.			Urea N, 10 hrs.		
		Amount	N^{15} concentration	N^{15} excreted	Amount	N^{15} concentration	N^{15} excreted
	mg.	mg.	atom per cent excess	mg.	mg.	atom per cent excess	mg.
1	1.575	90.1	0.843	0.760	68.6	0.670	0.460
2	1.770	89.5	0.808	0.723	68.7	0.669	0.460
3	1.770	119	0.722	0.858	91.6	0.521	0.477
4	1.674	103	0.754	0.775	79.0	0.585	0.462
5	1.625	103	0.651	0.671	81.2	0.542	0.440

Rat No.	Ammonia N, 10 hrs.			Total N, 48 hrs.			N^{15} retained, 48 hrs.
	Amount	N^{15} concentration	N^{15} excreted	Amount	N^{15} concentration	N^{15} excreted	
	mg.	atom per cent excess	mg.	mg.	atom per cent excess	mg.	per cent
1	6.34	1.296	0.0825	294	0.354	1.040	34.0
2	6.07	1.280	0.0778	323	0.298	0.932	47.4
3	7.56	1.146	0.0867	370	0.277	1.025	42.1
4	8.72	1.297	0.1130	336	0.295	0.991	40.8
5	7.67	1.077	0.0826	346	0.259	0.897	44.8

TABLE II
Rates of Metabolism of Nitrogen of β -Alanine in Fasting Rats
Explanations of the symbols are given in the text.

Rat No.	k'_1	k_2	R	k_1
	per cent per hr.	per cent per hr.		per cent per hr.
1	8.66	4.44	0.605	5.24
2	7.85	7.05	0.636	5.00
3	10.50	7.60	0.556	5.84
4	9.04	6.16	0.596	5.39
5	7.63	6.17	0.656	5.00
Mean		6.28	0.640	5.29

Results

It may readily be seen from the data in Table I that in the intact fasting rat β -alanine undergoes extensive degradation. Appropriate calculations show that in 10 hours 27.4 per cent of the administered N^{15} appeared in the urinary urea and 5.3 per cent in the urinary ammonia, while 10.2

per cent was found in the non-urea non-ammonia fraction of the urinary nitrogen. It will be noted that 48 hours after injection of the amino acid an average of 41.8 per cent of the administered material had been retained.

Kinetic treatment of the data in Table I, by a method of calculation previously described (2), yields the results shown in Table II. Here k'_1 is the fraction of the nitrogen of β -alanine which appears in the urinary total nitrogen per hour; k_1 is the fraction of the nitrogen of the amino acid which is excreted as urea per hour; and k_2 is the fraction of the administered nitrogen which is presumed to be incorporated into peptide bonds per hour. If it is assumed that the kinetics of excretion of non-urea N^{15} are similar to those of urea N^{15} , it can readily be shown that $k_1 = k'_1 R$ where R is the ratio of urea N^{15} to total N^{15} . Thus the average value of k_1 is found to be 5.29 per cent per hour, almost twice the value found for L-aspartic acid² and glycine (2). It is also noteworthy that nearly identical values of k_2 have been observed for β -alanine, L-aspartic acid,² and glycine (2).

DISCUSSION

The rapidity of formation of urea from β -alanine is interpreted to indicate that this amino acid is more rapidly deaminated than either L-aspartic acid or glycine. This conclusion is based on evidence reported previously (2) that in the intact fasting rat the step which determines the rate of urea formation is concerned with deamination processes rather than with reactions involved in the Krebs-Henseleit cycle. Consistent with this view is the fact that differences in the rate of formation of urea from β -alanine, L-aspartic acid, and glycine have been elicited.

It should be noted in Table I that the isotope concentration of the urinary ammonia is greater than that of the urea, suggesting that β -alanine could not have undergone extensive conversion to aspartic acid prior to deamination. If this had not been the case, the relationship of the isotope concentrations of the ammonia and urea would have been reversed (3).

The observation that the rate of incorporation of the nitrogen of β -alanine into peptide bonds is the same as has been observed for α -amino acids raises the possibility that the step which determines the rate of synthesis of anserine and carnosine is determined by processes similar to those involved in the formation of the peptide bonds of proteins (2). However, until the extent of incorporation of β -alanine into anserine and carnosine is more specifically determined, this point will remain unclear, for it is evident that the isotopic label of β -alanine must have been widely distributed among those α -amino acids which are in equilibrium with the ammonia pool.

² To be reported.

SUMMARY

β -Alanine, labeled with N^{15} , was injected into fasting rats. It was found that the amino acid was extensively degraded. From measurements of the rate of excretion of urinary N^{15} , estimates were made of the rate of incorporation of the nitrogen of β -alanine into urea and into peptide bonds. The results of these calculations indicate that β -alanine is more rapidly deaminated than either L-aspartic acid or glycine, whereas the rate of incorporation of nitrogen into peptide bonds is the same for the three amino acids.

The authors are indebted to Miss Dorothy von Hacht for her capable technical assistance, and to Mr. Joseph B. Doolittle for performing the isotope analyses.

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EFFECTS OF FASTING AND GLUCOSE INGESTION ON THE RETENTION OF AMMONIA*

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Sprinson and Rittenberg,¹ in their studies of the effects of diet on the retention of ammonia, were led to conclude that ammonia is extensively utilized for protein synthesis only when there is a deficiency of dietary amino acids. For some time this laboratory has been engaged in a study of the influence of glucose on the metabolism of amino acids and proteins. The results so far obtained indicate that glucose causes a marked repression of amino acid catabolism.² These results led us to investigate the effects of glucose on the retention of ammonia, and the present communication reports the results of these studies. It was found that the retention of administered N¹⁵-ammonia was considerably less in fasting animals than in those fed glucose. Thus the conclusion that extensive utilization of ammonia occurs only when there is a deficiency of dietary amino acids does not appear to be entirely correct. Rather, the extensive retention of ammonia on a protein-poor diet, such as was used in the experiments described by Sprinson and Rittenberg, may at least in part be ascribed to the effects of the high carbohydrate content of the diet.

EXPERIMENTAL

Twelve male Sprague-Dawley rats, weighing 330 to 400 gm., were used. 24 hours before the administration of N¹⁵-ammonium citrate, food was removed from the cages of all animals, and the water bottles of six were replaced with an aqueous solution containing 30 per cent glucose. Ammonium citrate was prepared as a solution of the diammonium salt by distilling ammonia, containing 32.3 atom per cent excess N¹⁵, into the calculated amount of citric acid dissolved in water. The solution contained 1.72 mg. of nitrogen³ per ml.

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¹ Sprinson, D. B., and Rittenberg, D., *J. Biol. Chem.*, **180**, 707 (1949).

² To be reported.

³ Corrected for N¹⁵ content.

The ammonium citrate, 1 ml. per 100 gm. of body weight, was administered to the rats by intraperitoneal injection. For 10 and 48 hours after injection urine was collected. In order to assure the output of adequate volumes of urine, all rats were injected intraperitoneally with a 0.85 per

TABLE I
Excretion of Ammonia Nitrogen by Fasting Rats

Weight	Total N excretion		N ¹⁵ concentration		N ¹⁵ excreted		Excretion of fraction of N ¹⁵ fed	
	10 hrs.	48 hrs.	10 hrs.	48 hrs.	10 hrs.	48 hrs.	10 hrs.	48 hrs.
gm.	mg.	mg.	atom per cent excess	atom per cent excess	mg.	mg.	per cent	per cent
360	137	433	0.833	0.331	1.140	1.435	56.9	71.7
320	99	352	1.016	0.351	1.002	1.234	56.3	69.4
360	117	363	0.977	0.388	1.145	1.410	57.2	70.4
360	107	356	1.078	0.395	1.150	1.406	57.4	70.2
360	121	382	1.037	0.386	1.252	1.475	62.6	73.7
380	135	438	0.919	0.340	1.243	1.490	58.8	70.6
Mean							58.2	71.0

TABLE II
Excretion of Ammonia Nitrogen by Rats Receiving Glucose

Weight	Total N excretion		N ¹⁵ concentration		N ¹⁵ excreted		Excretion of fraction of N ¹⁵ fed	
	10 hrs.	48 hrs.	10 hrs.	48 hrs.	10 hrs.	48 hrs.	10 hrs.	48 hrs.
gm.	mg.	mg.	atom per cent excess	atom per cent excess	mg.	mg.	per cent	per cent
380	65	215	1.293	0.479	0.845	1.030	40.0	48.7
395	51	209	0.891	0.349	0.451	0.728	20.3	32.7
380	58	188	1.266	0.493	0.728	0.928	34.3	43.9
360	63	206	1.121	0.431	0.703	0.888	35.1	44.4
345	51	234	1.373	0.417	0.704	0.976	36.2	50.2
390	81	304	1.126	0.399	0.912	1.214	42.1	56.0
Mean							34.7	46.0

cent sodium chloride solution (1 ml. per 100 gm. of body weight) every 2 hours during the first 10 hours of each experimental day. At the termination of each period of collection, funnels and cages were rinsed, and the urine and washings made to suitable volumes. After filtration, aliquots were removed for quantitative and isotopic analyses of the urinary total nitrogen (micro-Kjeldahl).

During the 48 hours of the experiment the animals receiving glucose consumed an average of 100 ml. of the solution.

Results

In Tables I and II are recorded the data obtained. It is evident that the retention of administered ammonia was considerably lower in the fasting animals than in those receiving glucose. Although the fraction of the dose excreted by the fasting rats is less than that reported by Sprinson and Rittenberg for animals on a high protein diet, approximately twice as much N^{15} was excreted by fasting animals as was reported for animals subsisting on a low protein diet. Although the effect of glucose on the retention of ammonia was not as great as that resulting from the ingestion of a low protein diet, the excretion of N^{15} by fasting animals is seen to be more than 50 per cent greater than that exhibited by the glucose-fed rats.

SUMMARY

The effect of fasting and glucose ingestion on the retention of ammonia has been studied. It was found that the amount of N^{15} excreted by fasting animals receiving isotopic ammonium citrate is more than 50 per cent greater than that excreted by glucose-fed rats.

The authors wish to thank Miss Dorothy von Hacht for her excellent technical assistance and Mr. Joseph B. Doolittle for performing the isotope analyses.

THE INFLUENCE OF METHIONINE DEFICIENCY ON AMINO ACID METABOLISM IN THE RAT*

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The effects of a methionine deficiency upon liver enzyme systems in the rat have been reported in an earlier paper (1). Because of the striking relationship between a methionine deficiency and enzyme activity in the liver, it was decided to study the effects of this deficiency on the free amino acid content of tissues and the total amount of amino acids excreted in the urine and feces. Since the liver plays such an important rôle in general nitrogen metabolism, the free amino acid content of this organ was determined. The free amino acid content of the brain was determined to observe whether any correlation exists between its ability to retain protein, as pointed out by Addis *et al.* (2), and its free amino acid content during a methionine deficiency.

Microbiological procedures for the determination of amino acids in biological fluids have been used by many workers (3-21). The use of these methods for materials which may contain amino acids in combined forms, without preliminary hydrolysis, has been criticized (22-25) because of the utilization of some peptides in tungstic acid extracts of tissues by lactic acid bacteria. Nevertheless, such methods appear to be useful for studies in which comparative results are desired. Hier and Bergeim (5), when studying the amino acid content of plasma, showed that acceptable recovery values were obtained, that the values were reproducible, and that the results were not affected by nembutal anesthesia, by prolonged contact of the protein-free plasma with the precipitate, or by long storage of the filtrate at low temperature. Schurr *et al.* (15) reported the recovery values for amino acids to be acceptable when assaying plasma, liver, brain, muscle, and spleen for free¹ amino acids.

Bhatt (19), when assaying urine for its total amino acid content, showed the recovery values for the amino acids to be within the limits of error of the microbiological procedure.

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¹ "Free," as used throughout, implies microbiologically available amino acids and may include combined forms as well as free amino acids.

Because of the reported loss of appetite of animals on a methionine-deficient ration (26) a forcible feeding procedure was undertaken in order to observe the effects of a methionine deficiency without the influence of inanition.

EXPERIMENTAL

Care of Animals—Male, weanling rats of the Holtzman strain weighing approximately 40 gm. were used as experimental animals. They were fed a stock ration for 10 days, then placed in metabolism cages, and given a complete, purified ration *ad libitum* for 1 week prior to the forced feeding. This was done in order for the animals to become accustomed to the purified ration and to determine the average daily food intake. The purified ration employed was the best ration of Ramasarma, Henderson, and Elvehjem (27), and had the following composition in per cent: non-essential amino acids 7.9, essential amino acids 7.9, L-arginine monohydrochloride 0.5, glutamic acid 2, corn oil 5, Salts IV (28) 4, monobasic sodium phosphate monohydrate 0.63, dry vitamin mix² 2, and sucrose to make 100 per cent.

After transferring the animals to the metabolism cages, the daily food intakes were recorded and the urine and fecal collections were begun. The weights were recorded every 3 days and the urine and fecal collections were made every 4 days.

After the animals had been given the purified ration *ad libitum* for 1 week, they were divided into two groups of ten animals each. One group was forcibly fed a methionine-deficient ration having the same composition as the one described above, except that sucrose was substituted for methionine, while the other was forcibly fed the complete ration. To facilitate feeding, 77 gm. of the ration were homogenized with 38 ml. of water and given by stomach tube in three portions daily at 5 hour intervals. 3 ml., 2 ml., and 2 ml. portions were given at each feeding, respectively. This regimen supplied each animal with 6 gm. of dry ration daily, which was approximately the average daily consumption during the week of feeding *ad libitum*.

The level of 6 gm. daily was supplied throughout the period of forced feeding which was continued for 2 weeks. Since the animals receiving the complete ration gained weight constantly and appeared healthy at the termination of the feeding studies, it was believed that this amount was

² Each 2 gm. portion of the vitamin mixture in a sucrose base contained 0.2 mg. of thiamine hydrochloride, 0.3 mg. of riboflavin, 0.25 mg. of pyridoxine hydrochloride, 2 mg. of calcium *d*-pantothenate, 1.5 mg. of niacin, 100 mg. of choline chloride, 0.01 mg. of biotin, 0.02 mg. of pteroylglutamic acid, and 10 mg. of inositol. 2 drops of fortified halibut liver oil were administered each week by dropper.

sufficient to allow a good comparison between the animals receiving the methionine-deficient ration and those receiving the complete one. The animals receiving the deficient ration exhibited bleeding of the feet and mouth, a decrease in body weight, and death. The methionine-deficient condition appeared early during forced feeding, and a 40 per cent mortality in the methionine-deficient group was encountered in the 2 week period.

Preparation of Samples—The livers and brains were prepared in the manner described by Schurr *et al.* (15).

In order to obtain sufficient quantities of the liver and brain homogenates, samples from each group were pooled. The livers and brains from the methionine-deficient group consisted of two pools, with three animals in each pool; those from the control group consisted of three pools, with four, three, and two animals in each pool, respectively. The methods used for the assay of the liver and brain have been described by Schurr *et al.* (15).

The urine from each animal was collected in flasks containing toluene and hydrochloric acid. Three pools were made from each group at the termination of each collection period and diluted to an amount equivalent to 50 ml. per rat per day. The urine and feces from the same animals were pooled throughout the entire experimental period, except in the case of death of an animal. For the microbiological assays, 100 ml. of the diluted urine were dried *in vacuo* for 12 hours at 55°. Acid hydrolysis was carried out by adding 4 ml. of 3 N hydrochloric acid to the dried samples and autoclaving at 15 pounds pressure (121°) for 5 hours. The samples were then filtered, washed with hot water, adjusted to pH 7 with potassium hydroxide, and diluted to 50 ml. Dilutions of these hydrolysates were used for the microbiological determination of all the amino acids except tryptophan. Alkaline hydrolysis for the determination of tryptophan was carried out by adding 2 ml. of 5 N sodium hydroxide to the samples dried in the same manner as that previously described and autoclaving at 15 pounds pressure for 12 hours. The samples were then treated as above with hydrochloric acid for neutralization. The hydrolyzed samples were stored under toluene at -5° until the assays were carried out.

For the determination of arginine, histidine, and methionine in the urine from the methionine-deficient animals, the hydrolysates were diluted one and a half times; for the assay of tryptophan, the alkaline hydrolysates were diluted four times; for the assay of the remaining amino acids and methionine in the samples from the control animals, the acid hydrolysates were diluted two times.

The fecal collections were made at the same time as the urine collections and stored in 95 per cent ethyl alcohol acidified with hydrochloric acid.

In preparation for the amino acid determinations, the fecal samples were dried *in vacuo* for 12 hours at 55°, ground in a mortar, and stored in a desiccator at -5°. Each sample was divided into two equal portions for alkaline and acid hydrolysis. 10 volumes of 3 N hydrochloric acid were used for acid hydrolysis and 10 volumes of 5 N sodium hydroxide for alkaline hydrolysis; both were autoclaved, prepared, and stored in the same manner as the urine samples. To obtain a concentration of the amino acids which would be suitable for the assay, 1 gm. of the dried feces was diluted 400 times. The medium of Henderson and Snell (29) was used for all of the microbiological determinations. The following organisms were used for determining the amino acid composition of the urine and feces: *Lactobacillus delbrueckii* 3 for arginine, isoleucine, leucine,

TABLE I
Effects of Methionine Deficiency on Free Amino Acid Composition of Liver

Amino acid	Control group	Methionine-deficient group
	γ per gm. wet tissue	γ per gm. wet tissue
Arginine.. . . .	27.5 (27.1- 27.8)*	12.7 (11.8- 13.6)
Histidine.	23.6 (23.1- 24.9)	25.3 (24.0- 26.6)
Isoleucine.	48.1 (41.6- 52.2)	40.7 (39.2- 42.1)
Leucine.	60.9 (56.7- 65.5)	52.1 (51.4- 52.8)
Lysine.	96.8 (91.0-100)	120 (103 - 136)
Methionine	50.0 (45.5- 53.1)	33.2 (30.4- 36.0)
Phenylalanine	30.6 (25.9- 33.1)	22.4 (21.1- 23.6)
Tryptophan	14.1 (13.2- 15.3)	13.3 (13.3- 13.3)
Valine.	50.6 (47.9- 54.2)	45.4 (41.9- 48.9)

* The values represent the range of the pooled samples.

and valine; *Leuconostoc mesenteroides* P-60 for histidine, methionine, and lysine; *Lactobacillus arabinosus* for phenylalanine and tryptophan; and *Streptococcus faecalis* for threonine.

RESULTS AND DISCUSSION

Free Amino Acids in Liver and Brain—The results of the determination of the free amino acid content of the liver are presented in Table I. These results indicate that a methionine deficiency definitely brought about changes in some of the free amino acid concentrations in the liver, although the present knowledge of amino acid metabolism does not permit a conclusive explanation of the effects. The concentrations of arginine and methionine were decreased considerably in the liver of the methionine-deficient group, while those of isoleucine, leucine, and phenylalanine appeared to be decreased only slightly. Lysine was the only amino acid

whose concentration in the liver was increased by the methionine deficiency. The methionine content of the liver was decreased, possibly because of the lack of this amino acid in the ration. The reasons for the changes in the content of the free amino acids are not apparent at the present time. Thompson *et al.* (17) have shown that the free amino acid content of the liver does not follow any general pattern during fasting or nitrogen deprivation. The values obtained for the free amino acid content of the liver of the control group agree fairly well with those obtained by Schurr *et al.* (18), although some values varied considerably. This variation might be due to the relative size and strain of the animals.

The results of the free amino acid determinations of the brain presented in Table II indicate that, as in the liver, definite changes occurred as a result of the methionine deficiency. The content of arginine in the brain

TABLE II
Effects of Methionine Deficiency on Free Amino Acid Composition of Brain

Amino acid	Control group	Methionine-deficient group
	<i>γ per gm. wet tissue</i>	<i>γ per gm. wet tissue</i>
Arginine.....	47.2 (40.8-57.6)*	28.3 (23.8- 32.7)
Histidine.....	13.2 (12.6-13.9)	25.7 (24.4- 26.9)
Isoleucine.....	6.1 (4.3- 7.2)	5.2 (4.6- 5.8)
Leucine ...	11.9 (10.2-13.4)	14.8 (13.0- 16.6)
Methionine ..	10.9 (10.4-11.6)	10.6 (9.40-11.8)
Phenylalanine..	8.9 (8.0-10.2)	8.8 (8.0- 9.6)
Tryptophan ..	4.8 (3.9- 6.7)	2.3 (2.2- 2.4)
Valine..	13.9 (12.7-14.4)	10.6 (10.4- 10.7)

* The values represent the range of the pooled samples.

of the methionine-deficient group decreased considerably, following the same trend as in the liver. Although the concentrations of tryptophan and valine were also decreased in the brain, they did not change in the liver. The content of histidine in the brain of the methionine-deficient group increased greatly over that of the control group while in the liver its content did not change. The methionine content of the brain of the methionine-deficient group was the same as that of the controls. This should be contrasted to the decrease of methionine in the liver of the methionine-deficient animals. On the whole, the values for the free amino acid content in brain of the control group of animals agree favorably with those reported by Schurr *et al.* (18).

Amino Acid Excretion in Urine and Feces—Because very consistent values for the total amino acid content of the urine samples (Table III) were obtained throughout the dietary regimen, the values for the amino

acid content of all the pooled samples were averaged for the entire experimental period for both the methionine-deficient and the control group respectively. As shown in Table III, the methionine-deficient animals excreted considerably less methionine than the controls and a slightly lower amount of arginine, histidine, lysine, and phenylalanine. The animals receiving the complete ration excreted less valine than the methionine-deficient animals. The concentration of methionine in the urine was altered considerably. This might be expected since a methionine-deficient ration was being fed. Although Sauberlich *et al.* (21) were feeding a casein ration *ad libitum*, the values for the total excretion of amino acids in the urine of our control animals appear to agree favorably with those reported by them.

TABLE III
Effects of Methionine Deficiency on Total Amino Acid Content of Urine

Amino acid	Control group	Methionine-deficient group
	<i>mg. per rat per day</i>	<i>mg. per rat per day</i>
Arginine...	0.43 ± 0.04*	0.34 ± 0.03
Histidine	0.16 ± 0.01	0.12 ± 0.01
Isoleucine...	0.58 ± 0.06	0.62 ± 0.05
Leucine.....	0.59 ± 0.05	0.49 ± 0.05
Lysine.....	0.62 ± 0.05	0.52 ± 0.03
Methionine	0.28 ± 0.03	0.11 ± 0.01
Phenylalanine.....	0.62 ± 0.05	0.44 ± 0.04
Threonine	0.48 ± 0.03	0.47 ± 0.02
Tryptophan....	0.14 ± 0.03	0.09 ± 0.02
Valine.....	0.72 ± 0.08	0.97 ± 0.06

* The value represents the standard error of the mean.

The total amino acid content of the feces presented in Table IV indicates a considerable variation in the daily excretion of each amino acid from the control group as shown by magnitude of the standard errors. This variation can be partially explained on the basis of the variation in the total amount of feces excreted daily. Because of the high bacterial content of the feces, these values are probably indicative of their amino acid composition. It is evident, however, from comparison of the total nitrogen (30) and amino acid content of the feces of the two groups, that the methionine-deficient animals absorbed the amino acids as well as the controls. Although the amino acid content of the feces of the methionine-deficient animals appears to be lower in most cases, these differences are of doubtful significance.

The values for the per cent of the ingested amino acids excreted are

presented in Table V and indicate that a methionine deficiency does not cause an increase in the excretion of amino acids over that of the controls under the conditions of this experiment. Evidently, the animals are able to utilize the ingested amino acids, or at least retain them, as shown by

TABLE IV
Effects of Methionine Deficiency on Total Amino Acid Content of Feces

Amino acid	Control group	Methionine-deficient group
	mg. per rat per day	mg. per rat per day
Arginine..	0.95 \pm 0.18*	0.71 \pm 0.06
Histidine.....	0.20 \pm 0.03	0.18 \pm 0.01
Isoleucine.....	0.75 \pm 0.14	0.67 \pm 0.07
Leucine.....	0.94 \pm 0.22	0.82 \pm 0.07
Lysine.....	0.86 \pm 0.16	0.75 \pm 0.07
Methionine ..	0.35 \pm 0.07	0.30 \pm 0.03
Phenylalanine..	0.54 \pm 0.12	0.44 \pm 0.03
Threonine.....	0.80 \pm 0.17	0.74 \pm 0.07
Tryptophan.....	0.17 \pm 0.03	0.15 \pm 0.04
Valine.....	0.89 \pm 0.20	0.81 \pm 0.08

* The value represents the standard error of the mean.

TABLE V
Ingested Amino Acids Excreted in Urine and Feces

Amino acid	Control group	Methionine-deficient group
	per cent	per cent
Arginine.....	3.07	2.34
Histidine ..	1.12	0.94
Isoleucine...	2.21	2.15
Leucine.....	3.19	2.73
Lysine.....	1.97	1.70
Methionine..	1.75	
Phenylalanine.	2.15	1.63
Threonine....	2.13	2.02
Tryptophan....	2.54	2.00
Valine ..	1.92	2.12

comparison of the amounts excreted by the two groups. In a similar feeding experiment in which the nitrogen excretion was measured, the excretion of total nitrogen by the methionine-deficient group was approximately the same as that of the control group (30). This substantiates the fact that the amino acids were being retained by the methionine-deficient animals.

The forcible feeding employed in these experiments minimizes the effects which would have been caused by impairment of appetite due to the methionine deficiency. It is probable that considerably more variation in excretion of amino acid would have occurred if the animals had been fed *ad libitum* not only from variations in food intake but also from additional effects brought on by loss of appetite and consequent inanition. When more is known concerning general amino acid metabolism, changes which occur in the free amino acid content of tissues may be more easily interpreted.

SUMMARY

1. Rats were forcibly fed a methionine-deficient ration and the free amino acid content of the liver and brain and the total amino acid content of the urine and feces were determined.

2. The methionine contents of the liver and urine of the methionine-deficient animals were found to be lower than those of the control animals; however, the level of methionine in the brain and feces did not appear to be altered.

3. The arginine concentrations in the liver and brain of the methionine-deficient animals were considerably lower than those of the control animals.

4. The methionine deficiency brought about definite changes in the free amino acid content of the liver and brain, although the changes were not as marked as might be expected. The total excretion of amino acids in the urine and feces was very nearly the same as that of the control animals.

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CARBOHYDRATE CHARACTERIZATION

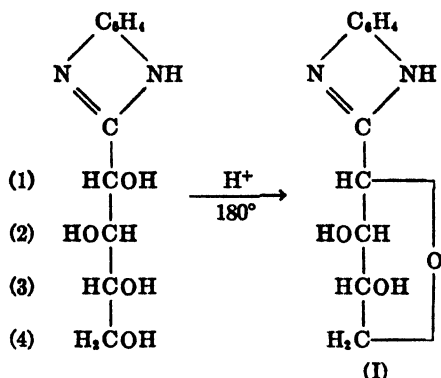
VI. SYNTHESIS OF AN ANHYDROALDOPENTOBENZIMIDAZOLE*

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In previous communications of this series (1, 2), it was reported that the pentose benzimidazole derivatives were converted to anhydro compounds (I) by heating at 180° in the presence of acid catalysts. These compounds were shown by degradation to possess a 1,4-anhydro ring structure (2), but the configuration of the carbon atoms, especially of carbon 1, remained unproved. Hence, it became desirable to establish by synthesis whether any inversion had occurred during anhydridization and to confirm the ring structures of anhydrides.

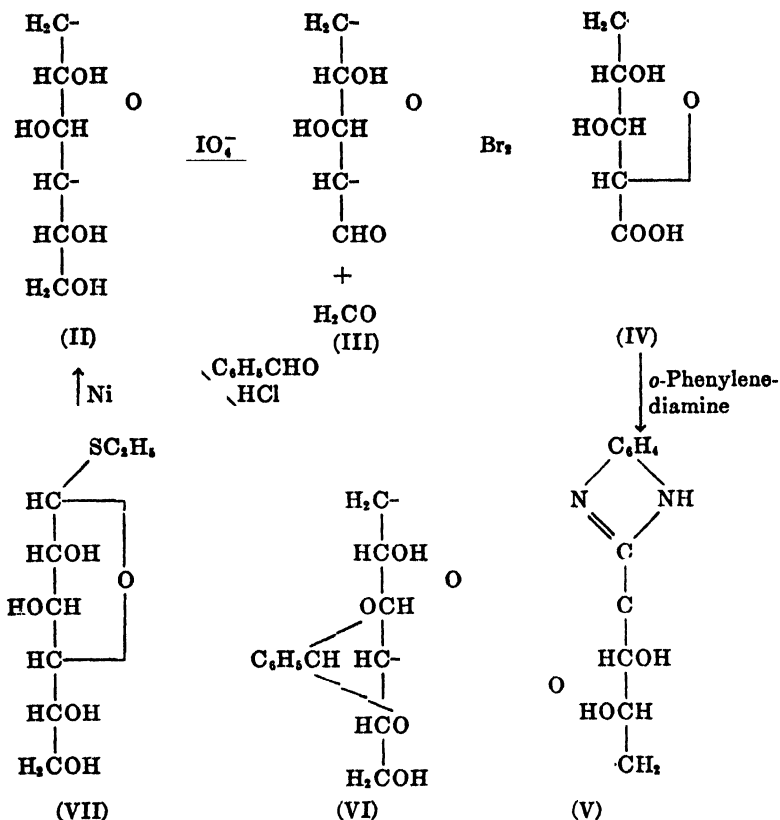


A compound of known structure containing the desired 1,4-anhydro ring is available in 1,4-sorbitan (arlitan) (II) (3, 4). This compound was selectively oxidized with 1 molar equivalent of periodate to produce 2,5-anhydro-L-xylose (III) and formaldehyde. III could be obtained only as a syrup, but the benzylphenylhydrazone could be crystallized. This hydrazone could be hydrolyzed in the presence of benzaldehyde to an analytically pure, syrupy product (III). Crude III was oxidized with

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bromine to the corresponding anhydropentonic acid (IV), which was then condensed with *o*-phenylenediamine at 135° to yield 1,4-anhydro-L-xylo-



benzimidazole (V), with the same melting point as that of product I, which was obtained by the anhydridization of D-xylobenzimidazole at 180°. Benzimidazole derivatives of the pentonic acids give no detectable amount of the anhydro product when subjected to these same conditions; *i.e.*, heating at 135° with the hydrochloric-phosphoric acid mixture for 2 hours. V had an optical rotation equal in magnitude to that of I, but was opposite in sign. Picrates of the two products also had the same melting point. This independent synthesis of an anhydroaldopentobenzimidazole is proof that formation of I takes place without inversion at carbon 1 during the anhydro ring formation.

To insure that the periodate oxidation of II would occur only at the desired position, an effort was made to protect the hydroxyls of carbons 2 and 3 by acetal formation. II, however, condenses with benzaldehyde in the presence of hydrochloric acid to produce the 3,5-benzylidene deriv-

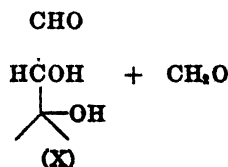
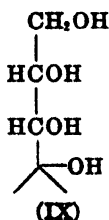
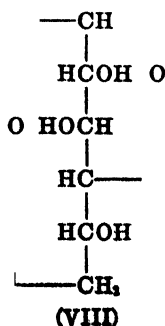
ative (VI) (m.p. 154–155°), which is apparently identical with the product obtained by Bashford and Wiggins (5) with the use of zinc chloride as catalyst. Since VI consumed no periodate and was oxidized by permanganate without loss of carbon to what is presumably 2,4-benzylidene-3,6-anhydro-L-gulonic acid, its structure is confirmed. Soltzberg *et al.* (3) described the preparation, in the absence of catalysts, of two monobenzylidene derivatives of 1,4-sorbitan (m.p. 136–140° and 121–122°), which are different from the isomer described here by us.

We have confirmed, by a synthetic approach, the structure given to 1,4-sorbitan by other workers (3, 4). This was accomplished by desulfurization with Raney's nickel (6–8) of α -ethylthio-D-glucofuranoside (VII) (9). The resulting compound was identical with II.

The selective oxidation by periodate or lead tetraacetate at a single 1,2-glycol grouping in compounds of the carbohydrate class, in which alternative positions of attack exist, has not been sufficiently explored. There are indications that this should be possible. First, it is known that *cis*-glycols are oxidized more rapidly than the corresponding *trans*-glycols (10). At one extreme is the *trans*-glycol, D-glucosan <1,4> β <1,6> (VIII), which has been reported by Dimler *et al.* (11) to be resistant to periodate. Secondly, if periodate esters are intermediates in the cleavage (10), the glycol grouping containing a primary hydroxyl should be preferentially attacked. These two points are illustrated in the oxidation of II to III by 1 molar equivalent of periodate and are reported here. With an excess of periodate, however, complete disruption of the carbon chain of II occurs (12).

The selective oxidation of a corticosteroid tetrol configuration (IX) to the dihydroxyaldehyde grouping (X) has been utilized by von Euw and Reichstein in a synthesis of Substance S (13).

We wish to acknowledge the gift of 1,4-sorbitan received through the late Dr. R. Max Goepp, Jr., Research Department, Atlas Powder Company.



EXPERIMENTAL

2,5-Anhydro-L-xylose (III) and 1,4-Anhydro-L-xylobenzimidazole (V) from 1,4-Anhydrosorbitan (II)—82 cc. of 0.30 M periodic acid (1 molar equivalent) were added dropwise over a period of 30 minutes to a stirred solution of 4 gm. of II in 25 cc. of water at 5°. After completion of the reaction, 1 molar equivalent of 0.4 M barium hydroxide was added. The precipitate was removed and the filtrate was oxidized by the bromine-barium carbonate method (14). After 12 hours the excess barium carbonate was removed and the barium ion in the filtrate was precipitated with the exact amount of sulfuric acid. The bromide ion was removed by shaking with silver carbonate and the silver was removed by hydrogen sulfide. The filtrate was concentrated *in vacuo* to a syrup which was heated at 135° with 2.6 gm. of *o*-phenylenediamine, 2.0 cc. of 85 per cent phosphoric acid, and 1.5 cc. of concentrated hydrochloric acid for 2 hours. The syrup was dissolved in 25 cc. of hot water, decolorized with Nuchar C, and made basic with ammonia. Two recrystallizations from water yielded 2.8 gm. of V; m.p. 225–228°; $[\alpha]_D^{25} = -64.6^\circ$ (*C*, 2.5; 5 per cent aqueous citric acid). The picrate was prepared, m.p. 190–192°. The recorded values (1) for anhydro-D-xylobenzimidazole are as follows: m.p. 224°; $[\alpha]_D^{25} = +64.8^\circ$ (*C*, 2.5; 5 per cent aqueous citric acid); the melting point of picrate was 191°. The melting point of a mixture of the two benzimidazoles and of the two picrates showed no depression.

The oxidation of 2 gm. of II was carried out as described above. After removal of the barium iodate, the solution was concentrated to a syrup *in vacuo*. The syrup was dissolved in 100 cc. of water and shaken with 4 gm. of benzylphenylhydrazine. After 2 hours, the semisolid mass was filtered and recrystallized twice from ethanol; yield 2.8 gm.; m.p. 180–181°; $[\alpha]_D^{27} = +28.0^\circ$ (*C*, 0.5 per cent in ethanol).

Analysis— $C_{18}H_{20}O_2N_2$. Calculated. C 69.2, H 6.5, N 9.0
Found. " 69.6, " 6.8, " 8.9

3.5 gm. of benzylphenylhydrazine of III were refluxed with 2 cc. of freshly distilled benzaldehyde in 100 cc. of water for 30 minutes. After extraction with ether, the aqueous phase was concentrated to a few cc. *in vacuo*. The remaining water was removed in a vacuum desiccator over phosphorous pentoxide to yield 1.2 gm. of an amber-colored syrup which could not be crystallized; $[\alpha]_D^{20} = -10^\circ$ (at equilibrium) (*C*, 1.2; H_2O).

Analysis— $C_8H_{10}O_4$. Calculated, C 45.5, H 6.1; found, C 45.4, H 6.0

3,5-Benzylidene-1,4-sorbitan (VI)—A stream of dry hydrogen chloride was rapidly passed through a suspension of 50 gm. of 1,4-sorbitan (II) in 75 cc. of benzaldehyde maintained at 0°. After about 10 minutes, the

mixture was saturated. The suspension was shaken at room temperature (about 1 hour) until complete solution and the subsequent crystallization of the benzylidene derivative had occurred. The mixture was kept in the ice box for 12 hours, filtered, and washed successively with Skellysolve B, dilute ammonia, and water. 50 gm. of crude VI, m.p. 120–130°, were obtained. Purification was achieved by recrystallization from ethyl acetate-Skellysolve B and from ethanol; yield 32 gm.; m.p. 154–155°; $[\alpha]_D^{30} = +17^\circ$ (C, 0.9; ethyl acetate).

Analysis— $C_{18}H_{18}O_4$. Calculated, C 61.8, H 6.3; found, C 62.2, H 6.3

On hydrogenolysis over palladium, VI yielded 1,4-sorbitan. No periodate consumption by VI in an ethanol-water mixture (1:1) was noted within 3 hours.

2,4-Benzylidene-3,6-anhydro-L-gulonic Acid—12.3 gm. of powdered potassium permanganate were added in small portions over a period of $\frac{1}{2}$ hour to a stirred suspension of 13.8 gm. of VI in 200 cc. of 1 per cent sodium hydroxide solution. The oxidation was complete in $\frac{1}{2}$ hour. The filtrate from the manganese dioxide was acidified and extracted with ethyl acetate. The ethyl acetate was removed *in vacuo* and the crystalline residue was twice recrystallized from ethanol; yield 4 gm.; m.p. 138–190°; $[\alpha]_D^{30} = +7.0^\circ$ (C, 1.2; ethyl acetate).

Analysis— $C_{18}H_{14}O_6$. Calculated. C 58.6, H 5.3, neutral equivalent 266
Found. " 58.5, " 5.3, " " 270

1,4-Sorbitan (II) from α -Ethylthio-D-glucofuranoside (VII)—VII (20 gm.) was refluxed with 100 gm. of Raney's nickel in 200 cc. of ethanol until the liquid phase was free of sulfur (36 hours). The centrifuged catalyst was washed thoroughly with ethanol and the combined ethanol solutions were concentrated to a syrup at 15 mm. The syrup was then distilled at 0.05 mm. and a bath temperature of 195–210°; 12 gm. were obtained; $[\alpha]_D^{25} = -13.6^\circ$ (C, 5.5; water). 3 gm. of the syrup yielded 1.0 gm. of 1,4-sorbitan (II) when recrystallized from isopropanol, m.p. 115–118°; $[\alpha]_D^{25} = -23.7^\circ$ (C, 1.2; water). When mixed with a sample of 1,4-sorbitan (m.p. 112–116°), $[\alpha]_D^{25} = -23.1^\circ$ (C, 2.0; water) no depression in melting point was observed.

SUMMARY

1. 1,4-Sorbitan was oxidized with periodic acid to 2,5-anhydro-L-xylose. The latter on oxidation to 2,5-anhydro-L-xylonic acid and condensation with *o*-phenylenediamine yielded 1,4-anhydro-L-xylobenzimidazole. This is proof that the anhydridization of the aldopentobenzimidazoles occurs without inversion.

2. 1,4-Sorbitan was converted to the 3,5-benzylidene derivative.

3. A synthesis of 1,4-sorbitan (arlitin) was achieved by the desulfurization of α -ethylthio-D-glucofuranoside.

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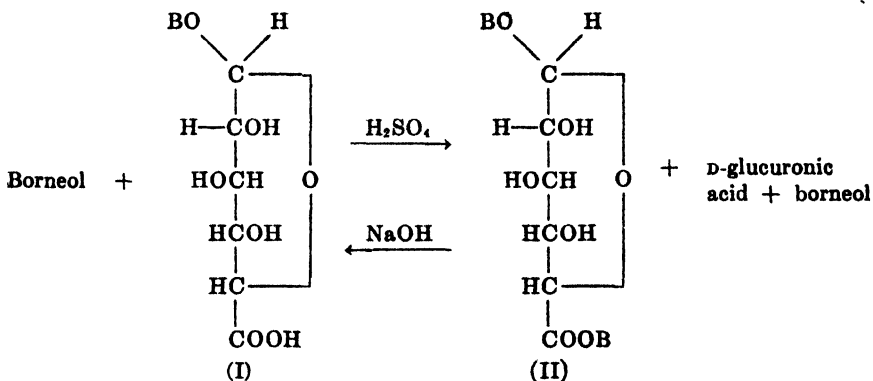
NOTE ON THE BORNYL ESTER OF BORNYL D-GLUCURONIDE*

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D-Glucuronic acid has been prepared in this laboratory during the past 10 years by the procedure of Miltzer (1) which is a modification of the method of Quick (2).



B = bornyl group

The essential difference in the Miltzer method is that the product isolated from the hydrolysis of bornyl D-glucuronide (I) is crystalline sodium glucuronate ($\text{C}_6\text{H}_9\text{O}_7\text{Na} \cdot \text{H}_2\text{O}$, calculated Na 9.8, found Na 9.9; $[\alpha]_D^{25} = +22.5^\circ$ (C, 2; water)), rather than the mixture of glucuronic acid and glucurone obtained by Quick. Steam distillation is carried out during the hydrolysis of I to remove the borneol formed.

We have consistently noted a small amount (about 5 per cent) of a resinous by-product remaining in the aqueous hydrolysate after the steam distillation. This resin yielded a neutral crystalline substance which proved to be the bornyl ester of bornyl D-glucuronide (II). It yields borneol and I on alkaline hydrolysis, thus indicating the points of attachment of the two borneol residues to the glucuronic acid molecule. Since I, obtained by alkaline hydrolysis of II, has the same melting point and

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rotation as the bornyl glucuronide isolated directly from dog urine, it appears probable that II is formed by esterification of unhydrolyzed I and not by the combined glycosidation and esterification of glucuronic acid during the acid treatment.

EXPERIMENTAL

300 gm. of I in 4 liters of boiling 0.2 N sulfuric acid were steam-distilled until no more borneol was collected (6 hours). The amber-colored droplets of the ester of bornyl D-glucuronide (II) suspended in the hot solution settled to the bottom on cooling and coalesced into a brown resin (15 gm.). This was dissolved in 25 cc. of 75 per cent boiling ethanol and, on cooling, partial crystallization occurred. After four recrystallizations from this medium and one recrystallization from an ether-Skellysolve B mixture, 1.2 gm. of the pure ester were obtained; m.p. 104–107°; $[\alpha]_D^{20} = -10^\circ$ (C, 1; ethanol). The sample was dried at 65° and 0.5 mm. over phosphorus pentoxide.

Analysis— $C_{24}H_{40}O_7 \cdot H_2O$. Calculated. C 64.4, H 9.1, mol. wt., (Rast) 484
Found. " 64.4, " 8.9, " " " 470

Two samples of the ester (200 mg.) were refluxed in a mixture of 15 cc. of 0.1 N sodium hydroxide and 25 cc. of methanol for 3 hours. Back titration with acid showed saponification equivalents of 470 and 485. On diluting the combined hydrolysates with water, 101 mg. of borneol were obtained. After sublimation, the melting point was 205–206°. No depression in the melting point of a mixture of this product and the borneol obtained directly from the original bornyl D-glucuronide (I) was observed. The filtrate, after the removal of the borneol, was acidified with 1 cc. of acetic acid, heated to 80°, and treated with an excess of zinc acetate. A precipitate of the characteristic zinc salt of I immediately resulted.

Analysis— $C_{16}H_{24}O_7 \cdot Zn \frac{1}{2} \cdot H_2O$. Calculated, Zn 8.6; found, Zn 8.4

1 gm. of this salt was converted to 0.45 gm. of the free acid (I) by the method of Murer and Crandall (3); m.p. 170–171° and $[\alpha]_D^{20} = -37^\circ$. The melting point of the bornyl D-glucuronide (I) isolated from the urine was 170–171° and $[\alpha]_D^{20} = -38^\circ$.

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THE MECHANISM OF THE BUTANOL-ACETONE FERMENTATION

I. THE RÔLE OF PYRUVATE AS AN INTERMEDIATE

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The question whether pyruvic acid holds a key position as an intermediate in the butanol-acetone fermentation by *Clostridium acetobutylicum* (Weizmann) similar to that in the alcoholic fermentation by yeast and in glycolysis by muscle has, so far, not received a decisive answer. This is mainly due to the inconsistent results obtained by different workers in this field. As in the case of corn-meal and glucose, the dissimilation of pyruvic acid should lead to the same products, viz., butanol, butyric acid, ethanol, acetic acid, acetone, and isopropanol respectively. A number of authors, Johnson *et al.* (1), Langlykke *et al.* (2), Brown *et al.* (3), Osburn *et al.* (4), and Davies *et al.* (5, 6), did obtain butyl products and acetone from pyruvic acid as the sole substrate or from mixed fermentations with glucose, but the yields were poor. On the other hand, Koepsell and Johnson (7, 8) and Simon (9, 10), using dried or washed cell preparations of *Clostridium butylicum* or *C. acetobutylicum*, found acetic acid to be the only dissimilation product, even in the presence of glucose.

Acetic acid was added as a labeled compound to fermenting corn mash. Wood, Brown, and Werkman (11) thus found that both isotopic butanol and acetone were formed in good yields. These authors suggested, therefore, that both butanol and acetone are derived from a common precursor and discussed the possibility of the following sequence of reactions: acetate \rightarrow pyruvate \rightarrow butyl products and acetone.

The regular formation of ethanol, a normal and independent dissimilation product in the butanol-acetone fermentation, stresses the importance of pyruvic acid as a possible intermediate. Its rôle as precursor of butyl products and acetone made a reinvestigation desirable.

The present paper deals with the study of the experimental conditions which lead from pyruvate to butyl products and acetone with yields comparable to those obtained from both corn mash and glucose.

EXPERIMENTAL

Growth Medium—The medium was prepared according to Davies and Stephenson (5). The pH was adjusted to 5.8 to 6.0.

Culture and Inoculations—A tube containing a 24 hour-old, vigorously

fermenting culture of *C. acetobutylicum* (Weizmann) in 5 per cent corn mash was inoculated into 250 ml. of the growth medium. After 24 hours incubation at 37°, this fermenting mash was inoculated into 1250 ml. of medium and incubated for 15 hours at 34–35°. The bacteria were then centrifuged and suspended in freshly sterilized distilled water. After allowing CO₂ to pass through the bacterial suspensions for 2 minutes, they were *immediately* used for the experiments. Davies (5, 6) has demonstrated, and we have been able to confirm it,¹ that the activity of the bacterial suspension towards glucose decreases to zero 3 to 5 hours after harvesting. In order to achieve complete fermentation of our substrates within 5 hours we have chosen the amount of bacteria as follows: from the rate of fermentation, the amount of bacteria capable of fermenting 1 mm of glucose in 1 hour at 36° can be calculated (Weizmann and Rosenfeld (12))²; per mm of substrate, we have used the harvest of 150 to 300 ml. of glucose medium corresponding to 200 to 400 mg. of dry bacteria. More bacteria were used in special cases which will be described below.

Pyruvate—Pyruvic acid was prepared (13) and transformed into its sodium salt by the method of Lipmann (14).

Fermentation Experiments—According to need, the fermentation experiments were carried out in 50 ml. long-necked flasks connected with gasometers, as described by Weizmann and Rosenfeld (15), in Warburg vessels for kinetic measurements, or in Krebs vessels for the study of fermentation products of slow fermentations requiring the maintenance of artificial anaerobiosis.

Procedure and Methods—After completion of the fermentation, the mixtures were deproteinized with tungstic or trichloroacetic acid. Aliquots of the filtrates were submitted to alkaline distillation. Acetone was estimated iodometrically; isopropanol, butanol, and ethanol were oxidized by dichromate according to Stahly, Osburn, and Werkman (16). The residues of the alkaline distillations were acidified with H₂SO₄ and submitted to steam distillation with the addition of MgSO₄, as recommended by Friedemann (17). The distillates were aerated to remove CO₂ and titrated for total acidity with phenol red as an indicator. The sodium salts containing a slight excess of NaOH were evaporated to dryness. The method of chromatographic analysis of the butyric and acetic acids by Elsdén (18)³ was then followed. It was observed empirically that the

¹ Rosenfeld, B., and Simon, E., unpublished results.

² See Weizmann and Rosenfeld (12), p. 1388.

³ Elsdén has observed that occasionally the indicator was more strongly adsorbed by different samples of silica gel. We have found that with the same sample of silica gel the dye is better adsorbed if the ratio of silica gel to alkaline dye is adjusted to result in a yellow-green to green column rather than a bluish green to blue one. The optimum inside diameter of the column is 10 mm.

separation of butyric from acetic acid is complete if the band caused by the latter occupies 65 per cent of the total column.

Fermentation of Corn Mash and Glucose

The figures for the yields of fermentation products from corn-meal usually refer to those of a 5 to 6 per cent mash (19). The substrate concentrations generally used in the studies on intermediates are as a rule much lower. Therefore, basic fermentations of corn mash and glucose were carried out at concentrations comparable to those used later on with pyruvate. For the calculation of the results we have adopted the "glucose

TABLE I

Comparison of Fermentation Products from 5 and 1.2 Per Cent Corn Mash and 0.96 Per Cent Glucose Medium

Column 1 contains known (19) data recalculated as glucose equivalents. Column 2 represents the results of fermenting 1 liter of 1.2 per cent corn mash corresponding to 0.86 per cent glucose, inoculated with 10 ml. of a vigorously fermenting culture of *C. acetobutylicum*. Column 3 shows the result of fermenting 191 mg. of glucose with 10 ml. of freshly prepared bacterial suspension corresponding to 420 mg. of dry weight. Total volume, 20 ml. Temperature, 35°. pH 5.0. Duration of the fermentation, 1 hour.

Fermentation products	Glucose equivalents per mm glucose fermented		
	(1)	(2)	(3)
Acetone	0.310	0.301	0.272
Isopropanol		0.053	0.070
Butanol	0.487	0.328	0.320
Butyric acid	0.085	0.084	0.021
Ethanol	0.065	0.155	0.156
Acetic acid	0.029	0.149	0.092

* Butyl products.

equivalents" proposed by Johnson *et al.* (1).⁴ Table I shows that the yield of "butyl products" (glucose equivalents of butanol + butyric acid) at the lower substrate concentration is decreased.

Fermentation of Pyruvate and Influence of Potassium

The fermentation of pyruvate was carried out in the manner already described for glucose (Table I, Column 3).

Koepsell and Johnson (7) found that the addition of phosphate increased the rate of pyruvate breakdown with their enzyme preparation from *C. butylicum*. It was of interest to learn whether such an addition

⁴ See foot-note 1 (1), p. 152.

to the above basic experiments with pyruvate would influence the course of the fermentation, and, in particular, the composition of the fermentation products. As shown in Table II, Column 5, this was found to be the case: the amount of acetone formed increased in the presence of potassium phosphate. Further experiments, designed to prove the influence of phosphate, however, showed, contrary to expectation, that the beneficial influence observed with potassium phosphate is caused by the potassium ion alone; added potassium sulfate and chloride (Table II, Columns 3 and 4) produced the same increase of acetone as potassium phosphate. Sodium phosphate alone caused a slight increase of acetone (Column 6); we feel, however, that it is not justified to interpret this increase as a "phosphate effect." The identical results obtained with KH_2PO_4 and K_2SO_4 or KCl suggest that phosphate (introduced with the bacterial suspension) is

TABLE II
Comparison of Acetone Yields As Glucose Equivalents Per 2 mm of Fermented Pyruvate, Influence of Potassium

Bacterial suspensions corresponding to 420 mg. of dry weight were added to a freshly prepared solution of sodium pyruvate containing 2 mm. The volume was made up to 20 ml. with distilled water. The pH of the mixture was 5.0. The fermentation was complete after 3 hours at 35°.

Additions	None	K_2SO_4	K_2SO_4	KCl	KH_2PO_4	NaH_2PO_4	NaH_2PO_4 + K_2SO_4 (7)
	(1)	(2)	(3)	(4)	(5)	(6)	(7)
K^+ concentration, M	0.0	0.04	0.2	0.2	0.2	0.0	0.2
PO_4^{3-} " "	0.01	0.01	0.01	0.01	0.2	0.2	0.2
Acetone	0.128	0.178	0.219	0.204	0.202	0.157	0.190

present in adequate concentration at 0.01 M, the average inorganic phosphate concentration found by analysis of the media when no phosphate has been added. If, in addition to sodium phosphate, potassium sulfate was added, a value comparable with that of potassium phosphate alone resulted (Column 7).

As can be seen from Table III, the influence of added potassium phosphate is limited to an increased acetone with a corresponding decrease of acetate formation.

Influence of Magnesium

In view of the fact that potassium ions exert a beneficial influence upon the formation of acetone from pyruvate, the action of other cations in this respect was of interest. Since Mg^{++} is known to be an essential constit-

uent in a number of enzyme systems involved in the carbohydrate breakdown, we have tested the influence of magnesium ions alone and in combination with potassium phosphate upon the dissimilation of pyruvate.

Table IV, Column 1, shows that addition of Mg^{++} alone produces an increase of acetone which is of the same order as that observed with K^+

TABLE III

Total Fermentation Products As Glucose Equivalents Per 2 mm of Fermented Pyruvate, Influence of Potassium

Each flask contained 5 ml. of 4.4 per cent sodium pyruvate and 5 ml. of bacterial suspension corresponding to 420 mg. of dry weight. Total volume, 20 ml. pH 5.0. Temperature, 35°. Duration, 3 hours.

Fermentation products	2 mm pyruvate (1)	2 mm pyruvate and 4 mm KH_2PO_4 (2)
Acetone.....	0.128 \pm 0.033	0.202 \pm 0.030
Butanol.....	0.021 \pm 0.011	0.015 \pm 0.010
Butyric acid.....	0.164 \pm 0.029	0.132 \pm 0.032
Ethanol.....	0.100 \pm 0.039	0.082 \pm 0.038
Acetic acid.....	0.540 \pm 0.054	0.500 \pm 0.046
No. of independent runs....	7	9

TABLE IV

Total Fermentation Products Given As Glucose Equivalents Per 2 mm of Fermented Pyruvate, Influence of Magnesium

Each flask contained 5 ml. of 4.4 per cent sodium pyruvate and 5 ml. of bacterial suspension corresponding to 420 mg. of dry weight and the additions quoted in the table. Total volume, 20 ml. pH 5.0. Temperature, 35°. Duration, 7 hours.

Fermentation products	2 mm $MgSO_4$ (1)	4 mm KH_2PO_4 + 0.06 mm $MgSO_4$ (2)	4 mm KH_2PO_4 + 0.4 mm $MgSO_4$ (3)	4 mm KH_2PO_4 + 2 mm $MgSO_4$ (4)*	4 mm KH_2PO_4 + 4 mm $MgSO_4$ (5)
Acetone.....	0.210	0.204	0.224	0.306 \pm 0.023	No fermentation
Butanol.....	0.070	0.020	0.029	0.019 \pm 0.019	
Butyric acid.....	0.123	0.134	0.128	0.144 \pm 0.012	
Ethanol.....	0.143	0.086	0.107	0.096 \pm 0.013	
Acetic acid.....	0.393	0.539	0.533	0.455 \pm 0.025	

* The figures in this column represent the mean values of five runs.

(Table II). Addition of Mg^{++} in combination with potassium phosphate shows at the optimum concentration of 2 mm (Column 4) a further increase. Higher concentrations of Mg^{++} are inhibitory (Column 5). As in the case with potassium phosphate, the influence of Mg^{++} is limited to an increase of acetone and a corresponding decrease of acetate formation.

Mixed Fermentation of Glucose and Pyruvate

Although the yield of acetone was increased by more than 100 per cent upon the combined addition of K^+ and Mg^{++} , not the slightest analogous response was observed with those fermentation products involving hydrogenation, *viz.*, the butyl products apart from ethanol and isopropanol. It was assumed that the formation of butyl products depends to some extent upon hydrogen donated during the dehydrogenation of the triosephosphates in the initial stages of glucose fermentation. Mixtures of glucose and pyruvate were therefore expected to give increased yields of butyl products from pyruvate. As can be seen, however, from the results quoted in Table V, Column 3, and the corresponding calculated values (Column 4), the increase of butyl products at this ratio of glucose

TABLE V

Total Fermentation Products Given As Glucose Equivalents Per 1 mM of Glucose or 2 mM of Fermented Pyruvate

Each flask contained 5 ml. of bacterial suspension corresponding to 210 mg. of dry weight and 0.5 mM of KH_2PO_4 . Total volume, 20 ml. pH 5.0. Temperature, 35°. Duration, 3 hours.

Fermentation products	1 mM glucose (1)	2 mM pyruvate (2)	0.5 mM glucose + 1 mM pyruvate (3)	Calculated from $\frac{1}{2}(1) + \frac{1}{2}(2)$ (4)
Acetone	0.270	0.150	0.214	0.210
Isopropanol	0.091	0.014	0.027	0.052
Butanol	0.283	0.044	0.102	0.163
Butyric acid	0.047	0.116	0.173	0.082
Ethanol	0.168	0.055	0.098	0.111
Acetic acid	0.091	0.692	0.378	0.392

* Butyl products.

to pyruvate is small and not beyond the margin of experimental error. The results obtained are similar to those reported by Simon (10) using saline-washed bacteria.

*Influence of Fluoride upon Relative Amount of Hydrogen in
Fermentation Gases*

The failure to obtain an efficient hydrogen transfer in our coupled glucose-pyruvate fermentations may have been due to the absence or lack of an active phosphate transfer system in the bacterial suspensions applied.

If, in our case, hydrogenation is coupled with phosphate transfer, as demonstrated by Meyerhof *et al.* (20) in the glycolytic system, then the increase of the concentration of the phosphate-transferring system or its stabilization should lead to an increase of the hydrogenation reactions.

Fluoride has been successfully applied by Potter (21) as a stabilizer of high energy phosphate in kidney homogenates. We have studied the influence of fluoride upon hydrogen formation during pyruvate dissimilation with suspensions of *C. acetobutylicum*. Any decrease of hydrogen pro-

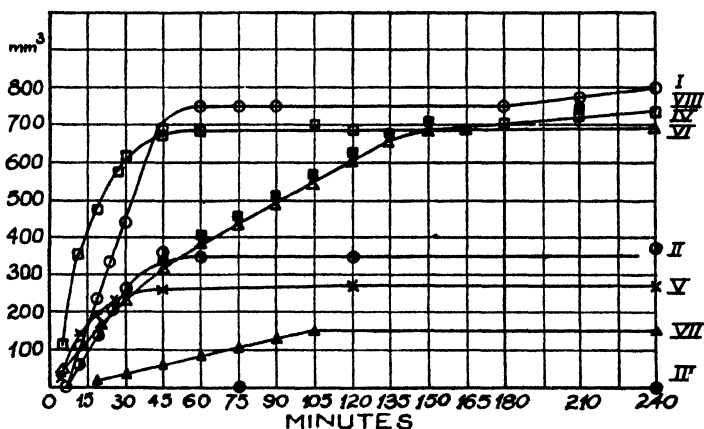


FIG. 1. Fermentation of glucose and pyruvate. In the main compartment of a series of three 30 ml. Warburg vessels, 0.2 ml. of 0.05 M glucose was placed, the center cup of Vessel 2 contained in addition 0.3 ml. of 1.1 M KOH and a strip of folded filter paper, and to Vessel 3 0.1 ml. of 0.4 M NaF was added. The volume was made up to 1.2 ml. with distilled water. The side bulb contained 1 ml. of bacterial suspension (89.5 mg. of dry weight per ml.). Each of the main compartments of Vessels 4 to 8 held 0.5 ml. of 0.04 M sodium pyruvate and 0.05 ml. of M KH_2PO_4 , the center cup of Vessels 5 and 7 contained 0.3 ml. of 1.1 M KOH for CO_2 absorption, and to Vessels 6 and 7 was added 0.1 ml. of 0.4 M NaF. The volume was made up to 1.1 ml. The side cup contained 0.1 ml. of 0.15 M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 ml. of bacterial suspension (89.5 mg. of dry weight per ml.). One run (Vessel 8) contained, apart from pyruvate and fluoride, 0.2 ml. of 0.05 M glucose. The corresponding blanks were allowed to run concurrently. The vessels were gassed with hydrogen and, after attainment of equilibrium, the contents of the side bulbs were mixed with that of the main compartments. Temperature, 33°; pH (initial) 5.0. Curve I, glucose, total gas (○); Curve II, glucose, hydrogen (●); Curve III, glucose + NaF (●); Curve IV, pyruvate, total gas (□); Curve V, pyruvate, hydrogen (×); Curve VI, pyruvate + NaF, total gas (Δ); Curve VII, pyruvate + NaF, hydrogen (▲); Curve VIII, pyruvate + glucose + NaF, total gas (■).

duction observed would indicate an increased hydrogen consumption for hydrogenation reactions.

The experiments were carried out in Warburg vessels. For comparison, experiments with glucose have been included.

The results of the fermentations, after correction for the corresponding blanks, are presented in Fig. 1.

Fig. 1 shows that, in distinction to the complete inhibition of glucose

fermentation in the presence of 0.02 M NaF (Curve III), pyruvic acid is fermented (Curve VI), though more slowly than in the absence of fluoride (Curve IV).

In the presence of fluoride, the percentage of hydrogen from pyruvate is lower (24.7 per cent of total gas, Curve VII) than without fluoride (38.5 per cent of total gas, Curve V). In confirmation of earlier data (19) we found 45.7 per cent hydrogen (Curve II) in the fermentation gas obtained from glucose. The rate of fermentation of pyruvate in the presence of fluoride is not influenced by the addition of glucose, Curve VIII being identical with Curve VI.

TABLE VI

Total Fermentation Products Given As Glucose Equivalents Per 3 mm of Fermented Pyruvate in Presence of Fluoride

The main compartment of a Krebs vessel (150 ml.) contained 15 ml. of bacterial suspension (1.20 gm. of dry weight), the side cup held 2.5 ml. of 0.4 M sodium pyruvate and 2 ml. of 2 M KH_2PO_4 , and in Vessel 2 additional 0.7 ml. of 0.6 M NaF. The gas phase was hydrogen. The temperature was maintained at 33°. pH 5.0. After attainment of equilibrium, the contents of the main compartment and the side cup were mixed. After 3 hours the fermentation ceased. The control experiments without fluoride were carried out in 50 ml. flasks with 5 ml. of bacterial suspension (0.40 gm. of dry weight) only. After 3.5 hours the fermentation was complete. All the results have been corrected with the corresponding blanks, and represent the mean values of three independent runs.

Fermentation products	Control (1)	0.02 M NaF (2)
Acetone.....	0.218 \pm 0.008	0.222 \pm 0.018
Butanol.....	0.013 \pm 0.001	0.062 \pm 0.032
Butyric acid.....	0.120 \pm 0.025	0.169 \pm 0.025
Butyl products.....	0.133 \pm 0.027	0.231 \pm 0.008
Ethanol.....	0.121 \pm 0.040	0.029 \pm 0.025
Acetic acid.....	0.533 \pm 0.031	0.380 \pm 0.014
Σ	1.005	0.862

Influence of Fluoride upon Composition of Fermentation Products from Pyruvate

The lower percentage of hydrogen observed with the manometric experiments suggested that in the presence of fluoride more hydrogenated products are to be expected from pyruvate than in the corresponding control experiments. These experiments have been repeated with 2 mm of pyruvate in order to establish a complete balance of the fermentation products.

Table VI shows that, although fluoride has no influence upon the amount of acetone formed, the butyl products are markedly increased. As can

be seen from the sum of the fermentation products, 0.14 glucose equivalents is not accounted for in the fluoride experiments.

DISCUSSION

Potassium has been shown by Davies (6) to be a limiting factor in the fermentation of corn mash. In view of our observation that both potassium and magnesium increase the yield of acetone from pyruvate to the same level as that obtained in a normal fermentation of both corn mash and glucose, it is strongly suggested that both elements play a rôle in the normal formation of acetone by the bacterial enzymes. In the glycolytic system the presence of both potassium and magnesium is required to catalyze the transformation of pyruvate to phosphoenolpyruvate (22). It is therefore considered a possibility that, in our case, acetone is formed from pyruvate via phosphoenolpyruvate.

The study of the requirement of external phosphate was rendered difficult because of the obligatory use of non-washed bacterial suspensions. The addition of our bacterial suspensions to the reaction mixtures thus resulted in a phosphate concentration of 0.01 M. This phosphate concentration is very close to that (0.01 to 0.02 M) with which Koepsell and Johnson (7) observed maximum activation of pyruvate dissimilation with extracts of *C. butylicum* under otherwise comparable experimental conditions. Therefore our failure to observe any influence of phosphate upon the pyruvate fermentation at still higher concentrations can, as yet, not be interpreted as suggesting the absence of phosphorylated intermediates. On the other hand, the influence of fluoride upon the pyruvate fermentation, leading to increased yields of butyl products, and the interesting observation made recently by Cohen-Bazire *et al.* (23) that arsenite suppresses the formation of butyrate from pyruvate can be considered as evidence favoring the assumption of phosphorylated intermediates.

The amount of bacterial suspension used appears to be unusually high; viz., 420 mg. of dry weight per 20 ml. as compared with 53 mg. of dry weight per 20 ml. by Davies (5, 6). In addition only one-sixth of the amount of substrate has been submitted to the action of the bacteria. Two considerations have prompted us to make use of large amounts of bacteria. (1) If we want to draw conclusions as to whether or not pyruvate is an intermediate, we have to study its dissimilation while the bacterial suspension shows maximum activity, i.e., within a few hours after harvesting. (2) The ratio of substrate to bacterial enzyme should at least approach that ratio which occurs under natural conditions; therefore, since the concentration of free pyruvate in equilibrium with the bacterial enzyme is presumably low, the concentration of the latter must be chosen as high as possible.

Working along these lines, we obtained acetone and butyl products in yields even higher than those reported by Davies (5, 6). By using washed or acetone-dried bacteria (9, 10), acetic acid was the only dissimilation product from pyruvate. The same result was obtained with an extract from frozen cells (7, 8). In view of our present results, these bacterial preparations have to be considered as incomplete enzyme systems.

We are now investigating the deficit in the balance sheet (Table VI) after the fermentation of pyruvate in the presence of fluoride.

SUMMARY

1. In the presence of both potassium and magnesium the yields of acetone from the dissimilation of pyruvate with bacterial suspensions of *Clostridium acetobutylicum* (Weizmann) are the same as those obtained from both corn mash and glucose.

2. In the presence of fluoride the formation of hydrogen from pyruvate is decreased and the formation of butyl products increased.

3. The significance of these findings has been discussed and it has been shown that both acetone and butyl products are formed from pyruvate.

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THE MECHANISM OF THE BUTANOL-ACETONE FERMENTATION

II. PHOSPHOENOLPYRUVATE AS A NEW INTERMEDIATE

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Since potassium and magnesium ions have been shown to favor the formation of acetone from pyruvate by suspensions of *Clostridium acetobutylicum* (Weizmann) (1) and since these cations are known to catalyze the transformation of pyruvate into phosphoenolpyruvate (2), there was a possibility that the latter may be an intermediate in the butanol-acetone fermentation. No attempt has so far been made to test the fermentability of phosphoenolpyruvate with butyl bacteria.

This paper deals with the study of the conditions required for the successful fermentation of phosphoenolpyruvic acid.

EXPERIMENTAL

The preparation of bacterial suspensions of *C. acetobutylicum* (Weizmann) was carried out with the same culture medium as that described in the preceding paper (1). *Phosphoenolpyruvic acid* was prepared from pyruvic acid and phosphorus oxychloride in the presence of quinoline essentially as described by Kiessling (3). According to his procedure for the purification of the barium salt, preparations of 70 to 80 per cent purity were obtained, containing little inorganic barium phosphate and the correct ratio of 1 atom of P, liberated by hypiodite, to 1 molecule of pyruvic acid. Prior to the experiments, the barium salt was transformed into the corresponding sodium or potassium salt.

Fermentation Experiments—In preliminary experiments we failed to achieve the fermentation of phosphoenolpyruvate, using the experimental conditions applied to the analogous fermentation of pyruvate (1). Even in the presence of phosphate acceptors, the substrate was not attacked and 90 per cent of the hypiodite-liberated phosphorus applied was found in the trichloroacetic filtrate of the fermentation mixture. The reason for this failure was the unfavorable ratio of substrate to enzyme. Meyerhof and Kiessling (4), for example, applied the extract of 8.8 gm. of dry yeast for the fermentation of 1 mM of barium phosphoenolpyruvate (742 mg.), whereas we applied only one-twentieth, i.e. 0.42 gm. of dry bacteria per 1 mM of substrate. We were, however, successful with 6 times the above

amount of bacteria, *i.e.* 2.5 gm. of dry bacteria. Higher amounts would have been impracticable because of the high blank values involved. Only in the Warburg experiments did we use an amount of bacteria equivalent to the amount of yeast used by Meyerhof and Kiessling (4); *i.e.*, 85 mg. of dry bacteria per 1×10^{-5} M of barium phosphoenolpyruvate. With this relatively high amount of bacterial suspension, the fermentation systems were not only supplied with an adequate amount of enzymes but, in addition, also with the required amount of specific phosphate acceptors for the high energy phosphate in the substrate.

The experiments, designed to give the complete balance of the fermentation products, were carried out in Krebs vessels.

Procedure and Methods—After completion of the fermentation, the mixtures were deproteinized and the estimation of the fermentation products was carried out as described for the analogous experiments with pyruvate (1). P estimations were made in the Warburg experiments according to the method of Berenblum-Chain, modified by Pons and Guthrie (5).

Fermentation of Phosphoenolpyruvate; Manometric Experiments

Preparation of Potassium Phosphoenolpyruvate—0.318 gm. of barium salt in a 15 ml. centrifuge tube was dissolved by adding 1.1 ml. of N HCl and 4 ml. of H₂O. Ba⁺⁺ was precipitated by adding 2.4 ml. of 0.5 M K₂SO₄, centrifuged, and washed twice with 3.5 ml. of H₂O, containing 1 drop of 0.5 M K₂SO₄ in order to prevent the formation of colloidal BaSO₄. The supernatant and washings were adjusted from pH 3.7 to 4.8 by the addition of approximately 0.3 ml. of M KOH. The volume was made up to 15 ml.

Analysis—0.5 ml. of this solution was made up to 20 ml. (Solution 1). 10 ml. of this dilution were treated with 6.25 ml. of 0.1 N iodine and 2 ml. of 7 N NaOH. After standing for 15 minutes at room temperature, the alkaline solution was acidified with 3 ml. of 5.5 N H₂SO₄, and excess iodine was titrated with 0.1 N thiosulfate without the use of starch as indicator. 0.80 ml. of 0.1 N iodine has been used, corresponding to 1.18 mg. of pyruvic acid. After addition of 1 ml. of excess 0.1 N thiosulfate, the solution was made up to 50 ml. (Solution 2).

Inorganic P—1 ml. of Solution 1 contained 2.3 γ of P.

Hypiodite-Liberated P + Inorganic P—5 ml. of Solution 2 contained 40.5 γ of P.

Therefore, after deducting the inorganic P, 0.5 ml. of the original stock solution contained 764 γ of P, corresponding to 9.1 mg. of pure barium salt, C₃H₄O₁₃P₂Ba₂, or 2.17 mg. of pyruvic acid. Direct titration yielded 2.36 mg. of pyruvic acid. The preparation applied was 75 per cent pure.

The following data show the keeping quality of the potassium phosphoenolpyruvate solution (pH 4.8) in the refrigerator at 2°: after 1 week no change, after 1 month 93.5 per cent of the initial value.

The results of the fermentation experiments, after correction for the corresponding blanks, are presented in Fig. 1. Curve I represents the

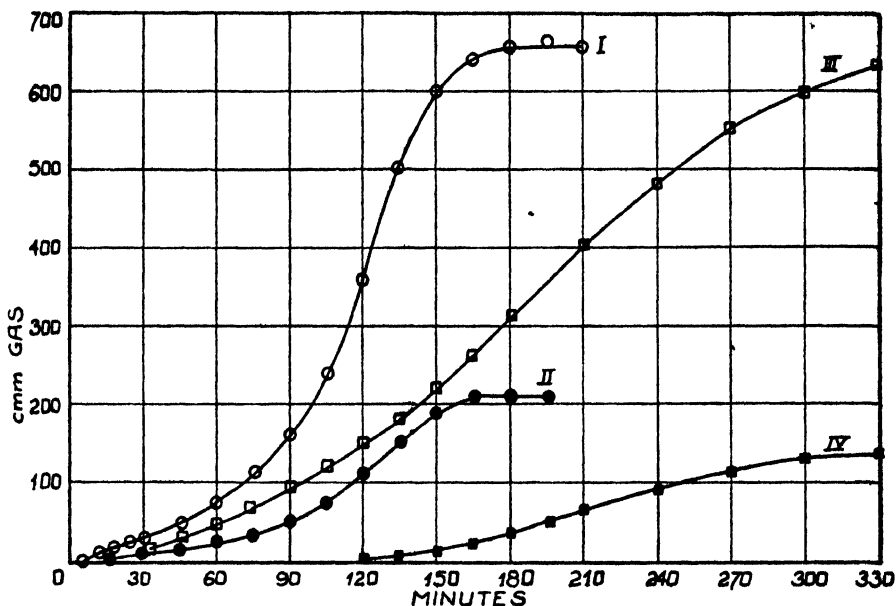


FIG. 1. Fermentation of phosphoenolpyruvate. In the main compartment of a series of four Warburg vessels 0.5 ml. of potassium phosphoenolpyruvate (764 γ of P) was placed. In addition, Vessels 3 and 4 contained 0.1 ml. of 0.4 M NaF. The center cup of Vessels 2 and 4 contained 0.3 ml. of 1.1 M KOH and a strip of folded filter paper for the absorption of CO₂. The total volume was made up to 1.2 ml. In the side cup 1.0 ml. of bacterial suspension was placed, each suspension containing 84.2 mg. of dry weight. Gas phase, H₂. Temperature, 33°. After attainment of equilibrium, the contents of the side bulbs were mixed with that of the main compartments. Curve I, phosphoenolpyruvate, total gas (○); Curve II, phosphoenolpyruvate, hydrogen (●); Curve III, phosphoenolpyruvate + NaF, total gas (□); Curve IV, phosphoenolpyruvate + NaF, hydrogen (■).

typical fermentation of phosphoenolpyruvate with the characteristic increase of fermentation velocity after an initial period of slow fermentation. In the presence of fluoride (Curve III), phosphoenolpyruvate is likewise fermented though more slowly.

The corresponding Curve II, representing hydrogen formation, indicates that in the absence of fluoride 32 per cent of the total gas was hydro-

In an analogous experiment 24 per cent of H_2 was observed. In comparison with the corresponding hydrogen value from pyruvate (38.5 per cent), these values are lower.

In the presence of fluoride (Curve IV), the amount of hydrogen formed is 22 per cent of the total gas. This value is low and of the same order as that which was observed with pyruvate in the presence of NaF. P estimations were made in the trichloroacetic acid filtrates after completion of the fermentation. No hypiodite-liberated P was found in the absence of fluoride; however, in its presence, on an average one-fifth remained.

TABLE I
Fermentation of Phosphoenolpyruvate

Fermentation products given as glucose equivalents per 2 mm of fermented phosphoenolpyruvate.

Fermentation products	No addition		0.02 M NaF	
	Experi- ment 1	Experi- ment 2	Experi- ment 1	Experi- ment 2
1. Acetone	0.139	0.115	0.259	0.300
2. Butanol.....	0.303	0.169	0.141	0.000
3. Butyric acid.....	0.006	0.220	0.032	0.138
4. Butyl products.....	0.309	0.389	0.173	0.138
	(0.349)*		(0.156)	
5. Fermentation products through conden- sation of substrate (1) + (2) + (3) ..	0.448	0.504	0.432	0.438
	(0.476)		(0.435)	
6. Ethanol.....	0.098	0.070	0.040	0.418
7. Acetic acid.....	0.467	0.403	0.368	0.304

* The figures in parentheses represent the mean value.

Fermentation of Phosphoenolpyruvate; Fermentation Products

Substrate—The barium salt used for these experiments was stored for 1 year in a desiccator at 2°. It contained 6.55 per cent of P liberated by hypiodite oxidation, the same value as that which was obtained a year ago, and corresponded to a purity of 78 per cent.

Preparation of Alkali Salt—The low solubility of K_2SO_4 in water did not permit the preparation of the potassium salt, as in the case of the Warburg experiments, since the volume of the substrate in the side cups of the Krebs vessels was limited to 6 ml. Therefore the following procedure was adopted. 1.60 gm. of barium salt in a 15 ml. centrifuge tube was dissolved in 6 ml. of N HCl. The Ba^{++} was precipitated with 2.2 ml. of a 3 M Na_2SO_4 solution (40°) and centrifuged. The barium sulfate was washed twice with 4 ml. of water and 1 drop of 0.5 M K_2SO_4 . The supernatant and the washings were adjusted from pH 3.0 to 4.8 by the

addition of 0.61 ml. of 5 N KOH. The total volume of the stock solution was 16 ml.

Procedure—The main compartment of a series of 150 ml. Krebs vessels contained 15 ml. of bacterial suspension (1.2 gm. of dry weight); the side bulbs contained 5.5 ml. of phosphoenolpyruvate stock solution corresponding to 35.3 mg. of P (Experiment 1) and 24.7 mg. of P (Experiment 2); in a parallel series 0.7 ml. of 0.6 M NaF was added to the substrate.

After gassing with hydrogen, the contents of the side bulb were added to that of the main compartment. Temperature, 33°; duration, 6 hours (Experiment 1) and 5 hours (Experiment 2). The fermentation products in an aliquot of the tungstic acid filtrates were analyzed as described previously (1).

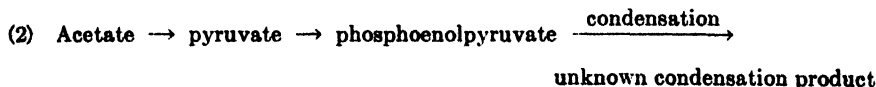
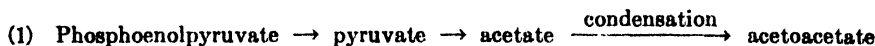
The results of Experiments 1 and 2 are shown in Table I.

Consistent with the low values of free hydrogen observed in the Warburg experiments (Fig. 1), high values of butyl products (0.349 mm) were obtained in the absence of fluoride. These values are higher than those obtained from pyruvate in the presence of fluoride (0.231 mm) (1).

In the presence of fluoride the "condensation products" (Table I) are of the same level (0.435 mm) as those without fluoride (0.476 mm); however, the course of the reaction is changed to favor acetone production.

DISCUSSION

The fermentation of phosphoenolpyruvate by suspensions of *C. acetobutylicum* (Weizmann) to acetone and butyl products opens new aspects regarding the mechanism of the butanol-acetone fermentation. The formation of acetone and butanol or butyric acid involves a condensation reaction as a characteristic feature. Both acetate (6) and pyruvate (1) and, as has been demonstrated in this paper, also phosphoenolpyruvate are capable of entering this condensation reaction. Obviously two reaction sequences exist which ultimately lead to this condensation.



Because of its smooth transformation into acetone by bacterial suspensions or enzyme preparations (7) acetoacetate has been considered to present the initial condensation product. However, attempts to demonstrate that acetoacetate can likewise be hydrogenated by bacterial suspensions to butyl products have not been successful (8). It is therefore still questionable, at least with our strain of *C. acetobutylicum*, whether or

not acetoacetate is the precursor of the butyl products. We believe that the inverse sequence of reactions, Reaction 2, offers a better possibility to explain both hydrogenation to butyl products and acetone formation. The enolic double bond, as well as the high energy phosphate, renders phosphoenolpyruvate particularly suitable to enter into self-condensation and to form an intermediate which upon a series of hydrogen transfers, dephosphorylations, and decarboxylations may yield both acetone and butyl products.

The low yields of butyl products from pyruvate have been explained by Johnson *et al.* (9) to be due to the degree of oxidation of pyruvate compared with that of glucose. Phosphoenolpyruvate, having the same level of oxidation as pyruvate, gave, however, higher yields of butyl products, thus indicating that apart from the degree of oxidation the latent energy content may play a rôle.

SUMMARY

1. Phosphoenolpyruvate is fermented by cell suspensions of *Clostridium acetobutylicum* (Weizmann).

2. The same fermentation products are formed as those from glucose and pyruvate.

3. The yield of butyl products from phosphoenolpyruvate is 2.6 times higher than that from pyruvate and 50 per cent higher than that from pyruvate in the presence of fluoride.

4. Fluoride decreases the fermentation velocity and favors acetone formation.

5. The rôle of phosphoenolpyruvate in the butanol-acetone fermentation has been discussed.

The authors wish to express their gratitude to Professor Ch. Weizmann for his constant interest and kind encouragement.

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THE METABOLISM OF THE ORGANIC ACIDS OF TOBACCO LEAVES

III. EFFECT OF CULTURE OF EXCISED LEAVES IN SOLUTIONS OF OXALATE

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Oxalic acid has long been regarded as an end-product of oxidative metabolic reactions in leaves. It is widely distributed in plants although the relative quantity present is not commonly great; nevertheless, in certain genera characterized by highly acid saps, such as *Oxalis*, *Rumex*, *Begonia*, and *Rheum*, oxalic acid may be the organic acid component present in greatest proportion. In tobacco, a species characterized by a high relative proportion of malic acid in the leaves, oxalic acid usually accounts for about 12 per cent of the total organic acids and for about 2 per cent of the organic solids; in the leaves employed for the present experiments, these figures were, respectively, 9.7 and 1.8 per cent.

When tobacco leaves are excised and subjected to culture in darkness with their bases in water, malic acid diminishes in concentration and citric acid increases. Evidence has been presented in earlier papers (1-3) which suggests that this is an expression of a normal series of metabolic reactions whereby approximately 2 moles of malic acid undergo chemical changes that result in the formation of 1 mole of citric acid. It has also been shown that, if organic acids are taken up by the tissues as potassium salts dissolved in the culture solution, marked effects upon the course and extent of this reaction can be demonstrated. Members of the Krebs tricarboxylic acid cycle are especially effective. Malic acid has been found to stimulate the reaction while citric acid appears to reverse it. It seemed desirable, therefore, to examine the effect of oxalic acid, the third most important organic acid component of tobacco leaves, to see whether any influence is exerted by this substance upon the metabolic reactions of the acids of the leaf cells.

EXPERIMENTAL

Tobacco plants (*Nicotiana tabacum*, var. Connecticut shade-grown), grown as previously described (3), were sampled by the statistical method (4) 57 days after being transplanted, five samples of twenty leaves each being taken from ten plants. Of these, one was at once dried for analysis

and the others were subjected to culture in a completely dark room, the temperature of which was controlled at 24° and the relative humidity at 50 per cent. The potassium oxalate solution on which two of the samples were cultured was 0.2 M in concentration and was adjusted to pH 6.05, 1490 ml. being used for each. The other two samples were cultured on water. Culture periods of 24 and 48 hours were arbitrarily adopted.

The oxalate solutions remaining were recovered for analysis as nearly quantitatively as possible; they were, respectively, at pH 6.7 after 24 hours and at pH 6.6 after 48 hours. There was no obvious evidence of the growth of microorganisms.

TABLE I

Effect of Culture on 0.2 M Potassium Oxalate upon Composition of Excised Tobacco Leaves

The figures not otherwise designated represent milliequivalents per kilo of original fresh weight of leaves.

	Control before culture	Changes during culture in darkness			
		Water, 24 hrs.	Water, 48 hrs.	Oxalate, 24 hrs.	Oxalate, 48 hrs.
Total nitrogen, gm.	4.79	+0.07	+0.14	-0.15	-0.13
Organic solids, gm.	75.6	-2.4	-3.1	-0.38	-0.79
Inorganic solids, gm.	17.6	+0.39	0.0	+11.7	+19.9
Alkalinity of ash.	352	-3.7	-3.5	+148	+249
Oxalic acid equivalent to alkalinity of ash.				+150	+253
Total organic acids.	307	-27	-31	+121	+220
Oxalic acid.	29.8	+0.2	+2.7	+164	+270
Citric "	41.5	+14.0	+23.8	+3.0	+22.9
Malic "	144	-8.6	-32.1	-5.9	-32.1
Undetermined acid.	90.9	-32	-25	-40	-41
pH of dry tissue	5.16	0.0	+0.07	+0.44	+0.63

The leaves cultured on water remained fully turgid and increased slightly in fresh weight; the leaves cultured on oxalate, on the contrary, became moderately flaccid within a few hours and had lost 34 per cent of their fresh weight after 24 hours and 59 per cent after 48 hours. However, there was no evidence of mottling until the 2nd day and it was then only slight.

The analytical results are shown in Table I, the methods mentioned in previous papers (3) being used.¹ The coefficient of variation of the nitrogen content was 2.6 per cent; the samples were therefore satisfactorily

¹ For the method to determine alkalinity of ash, see Vickery *et al.* (5).

constant in initial composition. The leaves were somewhat smaller and less well developed than those used for previous experiments (3) and the rate of respiration, as shown by the loss of organic solids from the samples cultured on water, was only about half as great.

Culture on Water—The alkalinity of the ash decreased by about 1 per cent, a negligible quantity, and the total organic acids by 9 per cent in 24 hours and 10 per cent in 48 hours. Although this determination is not highly accurate, the analytical error being of the order of 5 per cent of the quantity measured, the evidence suggests that there was a small loss of acids, possibly by respiration. Oxalic acid increased barely significantly. Citric acid increased and malic acid decreased in the normal manner. These particular leaves were therefore reliable experimental material to be used as controls for the experiment with oxalate, although they were appreciably lower in malic acid than usual. The undetermined acid diminished by about 10 per cent of the total acidity, and in an amount similar to the apparent loss of total organic acids. There was no significant change in the pH of the leaf extract.

Culture on Oxalate—The effect of the influx of potassium oxalate is evident in the smaller decrease of organic solids as compared with the leaves cultured on water and in the marked increase in inorganic solids. Furthermore, analysis of the residual culture solutions showed losses from them, respectively, of 10 and 13 gm. of oxalic acid per kilo of fresh leaves. That substantial quantities of the salt entered the leaves, in spite of their flaccid condition, is most clearly demonstrated by the increase in the alkalinity of the ash. The increase of 148 m.eq. in 24 hours is equivalent to 150 m.eq. of oxalic acid² at pH 6.0, the reaction of the culture solution, and that of 249 m.eq. in 48 hours is equivalent to 253 m.eq. The increases of oxalic acid found were, respectively, 164 and 270 m.eq.

The increases in total organic acids were somewhat smaller than those of oxalic acid, and, accordingly, the undetermined acid appears to have diminished. This change was probably significant, for the quantity of undetermined acid in the samples cultured on oxalate was only a little more than one-half that in the control sample analyzed at the start.

As in the samples cultured on water, citric acid increased and malic acid diminished, although the change does not seem to have been as extensive in the first 24 hours as it was in the control samples cultured on water. In 48 hours, however, the changes were quantitatively almost the same as in the controls. The pH of the tissues increased significantly as it has regularly been observed to do when salts of organic acids are absorbed by tobacco leaves from culture solutions.

² These figures are computed from the dissociation curves of oxalic acid.

The sample cultured for 48 hours on oxalate became greatly enriched in organic acids. The oxalic acid increased by a factor of 10 over the control and the total acids from 27.1 per cent of the organic solids to 37.8 per cent, if the undetermined acid is arbitrarily calculated as citric acid.

DISCUSSION

Oxalate absorbed from a culture solution by tobacco leaves in darkness does not appear to enter into the metabolic reactions to any significant extent. Within the errors of the methods, the analytical evidence indicates that the salt merely accumulates, for the increases in both cation and anion, expressed in equivalents, were essentially identical. Furthermore, the newly acquired oxalic acid exerted no detectable influence upon the behavior of the malic and citric acids. In the control leaves cultured for 48 hours on water, the gain in citric acid was 23.8 m.eq. (7.93 mm) and the loss of malic acid was 32.1 m.eq. (16.1 mm). The molar ratio of malic acid loss to citric acid gain was therefore 2.0 in agreement with previous results (3). In the leaves cultured for 48 hours on oxalate, the gain in citric acid was 7.63 mm, and the loss of malic acid 16.1 mm, the ratio being 2.1. Because of the small quantities of malic and citric acids which had undergone change at the end of 24 hours, no significance can be attached to the computed ratios of 0.9 and 2.9 for these two experiments, the errors in the analytical determination of malic acid being too great.

The only apparent influence upon the organic acids of culture on oxalate was that upon the undetermined acid. This quantity is computed by difference, and the analytical error is correspondingly large. However, there was a loss of nearly one-half of the undetermined acid during culture on oxalate for 48 hours, the analogous loss in the control experiment being only about one-quarter. No attempt to interpret this observation can be made until more information on the composition and behavior of the undetermined fraction of the organic acids has been obtained. It may, however, be connected with the stimulated respiration of these samples of leaves referred to below and thus may have no bearing upon the metabolism of oxalic acid.

Determinations of isocitric acid were made on all samples. Small negative quantities (of the order of -3 m.eq. per kilo) were found and presumably represent the error of the analytical method. This result confirms previous observations that tobacco leaves normally contain no readily demonstrable quantity of isocitric acid.

The present results do not conflict with the view that oxalic acid represents an end-product of oxidative metabolism in leaves. The reactions by which it is formed are clearly not of kinds that are reversed to any significant extent by the mere introduction of a large excess of oxalic acid for, if so, the increase in oxalic acid found in the sample cultured on

oxalate would not have been so closely equivalent to the increase in the alkalinity of the ash. Although no light has been shed upon the nature of the reactions whereby oxalic acid is synthesized in tobacco leaves, it seems clear that these reactions are not reversibly connected with those whereby malic acid is converted to citric acid. This is a further instance of the complexity of the metabolic systems in which the organic acids of leaves are involved.

The observed behavior of oxalic acid bears a close analogy to that of tartaric acid (2). This substance also appears to be freely taken up by tobacco leaves from a culture solution but, so far as present information goes, remains unchanged and exerts no detectable effect upon the metabolism of malic and citric acids.

A word should be added concerning the obvious increase in the respiration of tobacco leaves subjected to culture on potassium oxalate. The leaves lost 3.1 gm. of organic solids during culture on water for 48 hours in darkness. During culture on oxalate for a similar period, they lost 0.8 gm. but, at the same time, acquired 12.2 gm. of oxalic acid which was found as such in the tissues. Accordingly, the respiration must have been stimulated, for approximately 13 gm. of organic substance disappeared from the system. Analogous observations have been made with leaves cultured on malate, citrate, isocitrate, and acetate (3) and similar effects can be seen in the data of Pucher and Vickery (2) for several other organic acids. Turner and Hanly (6) have reported a marked increase in respiration when carrot root tissue slices were suspended in potassium chloride and especially in potassium succinate. Machlis (7) noted increases in the respiration of barley roots treated with potassium salts of malic, succinic, fumaric, and citric acids and also in the capacity of such roots to accumulate bromide ion, a function closely associated with the rate of respiration. However, similar results could be obtained with potassium sulfate, and he pointed out that the effects were probably due to the influx of potassium ions. The present observations may have a similar explanation.

SUMMARY

Leaves of tobacco (*Nicotiana tabacum*, var. Connecticut shade-grown) cultured for 48 hours in darkness on 0.2 M solutions of potassium oxalate accumulate this salt in considerable quantities. There was no effect, however, upon the conversion of malic to citric acid which normally takes place in such leaves in darkness, and no evidence was found to suggest that oxalic acid is connected by reversible equilibrium reactions with either of these metabolites. The results conform to the view that oxalic acid represents an end-product of oxidation reactions in this species.

Confirmation was obtained of previous observations that approximately

2 moles of malic acid are converted into 1 mole of citric acid during culture in darkness.

The respiration of the leaves, as estimated from the loss of organic solids during the culture period, was stimulated during culture on oxalate, possibly as a result of the presence of abnormal quantities of potassium ions.

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CYTOCHEMICAL STUDIES OF MAMMALIAN TISSUES

III. ISOCITRIC DEHYDROGENASE AND TRIPHOSPHOPYRIDINE NUCLEOTIDE-CYTOCHROME *c* REDUCTASE OF MOUSE LIVER

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An important aspect of the problem relating cell chemistry and cell structure is the intracellular distribution of the enzymes responsible for the reactions of the Krebs tricarboxylic acid cycle. Previous work has demonstrated that mitochondria play an important rôle in several phases of the cycle in that the succinoxidase and cytochrome oxidase activities of rat (1-3) and mouse (4) liver appear to be localized exclusively in these cytoplasmic elements. The complex system capable of oxidizing oxalacetic acid is also concentrated in the mitochondrial fraction of rat liver (5), although, in this case, several other fractions definitely enhance the activity of the mitochondria. The oxidation of citrate and α -ketoglutarate has been attributed to mitochondria (6), but data are not available showing to what extent the latter reactions are concentrated in the fraction as compared with the original whole tissue.

A major difficulty in attempts to study the intracellular distribution of the Krebs cycle reactions arises from the lack of direct methods of assay for many of the individual enzymes involved. Isocitric dehydrogenase is a notable exception, however, in that it can be readily determined by following spectrophotometrically the rate of reduction of triphosphopyridine nucleotide (TPN) in the presence of isocitrate (7, 8). TPN-cytochrome *c* reductase, an enzyme of importance in the Krebs cycle reactions since it presumably provides the link between isocitric dehydrogenase and the cytochrome oxidase system, can also be determined without difficulty. The present report includes a study of the distribution of these two enzymes in fractions isolated from C₃H mouse liver. Also included is a discussion of some of the difficulties involved in the interpretation of data obtained with the cell fractionation technique.

EXPERIMENTAL

Methods

Fractionation of Liver Homogenates—Livers were obtained from C₃H mice 2 to 4 months of age. The homogenates were prepared in both

0.25 M and 0.88 M sucrose and fractionated as described in a previous report (9). Enzyme assays were carried out on the original homogenates and on the following four fractions: nuclei (N_w), mitochondria (M_w), submicroscopic particles (P_w), and final supernatant (S_2). As noted previously (3), fraction N_w contained unbroken liver cells, red blood cells, and some mitochondria in addition to free nuclei. Fraction M_w was essentially cytologically homogeneous in that it contained relatively few cellular elements other than free mitochondria. Fraction P_w , which was optically empty in the light microscope, contained cellular particles sedimentable at high centrifugal forces ($130,000 \times g$ for 60 minutes in 0.88 M sucrose, $57,000 \times g$ for 60 minutes in 0.25 M sucrose). The final supernatant, fraction S_2 , contained soluble material and particles not sedimented at the forces used.

Determination of Isocitric Dehydrogenase—The determination of isocitric dehydrogenase was carried out spectrophotometrically at 24° by following the rate of reduction of TPN on addition of enzyme and *d*-isocitrate. The reaction mixtures contained 0.60 ml. of 0.050 M veronal- H_2SO_4 buffer, pH 7.4, 0.30 ml. of 0.82 M nicotinamide, 0.1 ml. of 0.018 M $MnCl_2$, water to a final volume of 3.00 ml., 0.050 to 0.300 ml. of a suitable dilution of each liver preparation, 0.30 ml. of 0.002 M KCN, 0.30 ml. of 0.0014 M TPN, and 0.10 ml. of 0.10 M potassium *d*-isocitrate. The blank contained all the reagents except the substrate. On addition of isocitrate, the reaction mixtures were transferred to cuvettes, and the increase in optical density at $340 m\mu$ was followed at 1 minute intervals in the Beckman spectrophotometer. The original homogenates and each fraction were assayed at two or more levels of enzyme activity.

The reaction mixture included nicotinamide to prevent enzymatic destruction of TPN by liver nucleotidase and KCN to inhibit reoxidation of dihydrotriphosphopyridine nucleotide ($TPNH_2$) through the TPN-cytochrome *c* reductase-cytochrome oxidase system. $MnCl_2$ was added to activate isocitric dehydrogenase (7, 8). Veronal was used instead of phosphate buffer because of the inhibiting effect of phosphates on the enzyme (7, 8). In all preparations assayed, the reaction proceeded linearly with time until the reduction of TPN was almost complete, and the rate of reaction was directly proportional to the concentration of the liver preparations.

TPN was isolated from hog liver according to the method of LePage and Mueller (10) and was further purified by counter-current distribution,¹ yielding a final product 68 per cent pure. The TPN content of

¹ In several experiments, TPN of approximately 50 per cent purity was obtained by the method of LePage and Mueller (10). When subjected to counter-current distribution in the system water-phenol (11) (eighteen to twenty-four transfers),

the preparation was determined by enzymatic reduction by employing an extinction coefficient at 340 $m\mu$ of 6.27×10^4 sq. cm. per mole for pure $TPNH_2$ (12).

The solution of potassium *d*-isocitrate was prepared by alkaline hydrolysis of dimethyl isocitrate lactone. The latter compound was isolated from the dried leaves of *Bryophyllum calycinum*³ according to the method of Pucher, Abrahams, and Vickery (13). After recrystallization from both water and ethanol, the lactone melted at 104–105° (uncorrected) and gave the following analysis: H 5.11, C 47.47 per cent; theory for $C_8H_{10}O_6$, H 4.99, C 47.52. The compound was hydrolyzed by adding a 20 per cent excess of KOH and heating the mixture at 100° for 1 hour in an open tube (to allow distillation of methanol). The solution was then neutralized to pH 7.4 with H_2SO_4 . The amount of KOH consumed during the hydrolysis was in agreement with the theory.

Determination of TPN-Cytochrome c Reductase—The TPN-cytochrome *c* reductase activity of the liver preparations was determined spectrophotometrically at 24° by following the rate of reduction of cytochrome *c* on addition of $TPNH_2$. The $TPNH_2$ was prepared by enzymatic reduction of TPN (68 per cent pure) and was kept as a stock solution which contained most of the components of the final reaction mixture used in the TPN-cytochrome *c* reductase determination. Each 30 ml. of the stock solution contained the following: 1.5 ml. of 0.50 M potassium phosphate buffer, pH 7.4, 1.5 ml. of 0.82 M nicotinamide, 10.5 ml. of H_2O , 3.0 ml. of an aqueous extract of hog heart acetone powder containing 0.01 M potassium *d*-isocitrate, 9.0 ml. of 0.002 M KCN, and 4.5 ml. of 0.0014 M TPN. The stock solution was allowed to stand at room temperature until the TPN was completely reduced (8 to 10 minutes) and was then chilled to 0°. No reoxidation or destruction of $TPNH_2$ occurred in 6 hours at 0°.

In carrying out the TPN-cytochrome *c* reductase determinations, 2.00 ml. aliquots of the stock $TPNH_2$ solution were equilibrated for 10 minutes in a water bath at 24°. Then to each tube were added the following: water to give a final volume of 3.00 ml., 0.050 to 0.300 ml. of a suitable dilution of each liver preparation, and 0.50 ml. of 2.44×10^{-4} M oxidized cytochrome *c*. The blank contained all components except the liver preparations. After the addition of cytochrome *c*, the increase in optical density at 550 $m\mu$ was followed at 1 minute intervals in the spectropho-

preparations of 68 to 75 per cent purity were obtained, and the TPN (distribution coefficient (K) = 1.2) was effectively separated from traces of DPN (K = 0.12 (11)). Attempts to increase the purity further by means of counter-current distribution have been unsuccessful.

³ The authors are indebted to Dr. H. B. Vickery for a generous supply of *B. calycinum* tissue.

tometer. The rate of reduction of cytochrome *c* by the liver preparations in the presence of TPNH₂ proceeded linearly with time and was directly proportional to the concentration of the liver preparations. All assays were carried out at two or more levels of enzyme activity.

The hog heart acetone powder was prepared according to the method of Green *et al.* (14) and was stored at -20° . The acetone powder extract was prepared by homogenizing the acetone powder in distilled water at 3° (1 gm. of acetone powder per 10 ml.) and centrifuging the homogenate at 50,740 r.p.m. for 30 minutes at 3° (Spinco ultracentrifuge, type A preparative rotor). The supernatant was dialyzed against distilled water at 0° for 6 hours, made 0.01 M with respect to potassium *d*-isocitrate, heated at $56-57^{\circ}$ for 15 minutes, and filtered.

The procedure of heating the acetone powder extract in the presence of isocitrate was found desirable for several reasons. A relatively large amount of inactive protein was denatured with little loss of isocitric dehydrogenase activity, and a considerable decrease occurred in the ability of the extract itself to reduce cytochrome *c* in the presence of TPNH₂. Although this blank was automatically compensated for in the reference cuvette, it was found to be troublesome when too high. When stored in the frozen state, the extract retained its isocitric dehydrogenase activity for more than a month.

Cytochrome *c* (Sigma Chemical Company) was dissolved in 0.005 N HCl. On reduction with hydrosulfite, the increase in optical density at $550\text{ m}\mu$ of the solution corresponded to extinction coefficients of 0.90×10^7 and 2.87×10^7 sq. cm. per mole for the oxidized and reduced forms.

Determination of Oxygen Uptake with Isocitrate As Substrate—In several experiments, a study was made of the ability of the liver preparations to take up oxygen in the presence of isocitrate. Reaction vessels chilled in cracked ice contained the following components: 0.10 ml. of 0.5 M potassium phosphate buffer, pH 7.4, 0.10 ml. of 0.82 M nicotinamide, 0.10 ml. of 0.018 M MnCl₂, 0.50 ml. of 2.44×10^{-4} M cytochrome *c*, 0.20 ml. of 0.025 M potassium adenosinetriphosphate (ATP), 0.3 ml. of 0.0014 M TPN, 0.30 ml. of 0.10 M potassium *d*-isocitrate, 0.100 to 1.00 ml. of the liver preparations in 0.25 M sucrose, sufficient 0.25 M sucrose to give a final sucrose concentration of 0.10 M, and water to give a final volume of 3.00 ml. The center wells contained 0.10 ml. of 2.5 N KOH. The vessels were equilibrated for 6 minutes at 38° in the Warburg apparatus and oxygen uptake measurements recorded at 5 minute intervals thereafter.

Phosphate instead of veronal buffer was used in this determination because the latter inhibited the aerobic reaction. Although ATP had no effect on the initial rate of the reaction, it was found that without ATP

the rate declined fairly rapidly after approximately 10 minutes. When ATP was included, the rate remained constant for approximately 30 minutes. The ATP used was obtained as the dibarium salt from the Sigma Chemical Company.

Results

Distribution of Isocitric Dehydrogenase—As shown in Table I, by far the majority of the total isocitric dehydrogenase activity of the homo-

TABLE I

Distribution of Isocitric Dehydrogenase and TPN-Cytochrome c Reductase in Mouse Liver Fractions

Preparation	Isocitric dehydrogenase			TPN-cytochrome c reductase		
	Total activity*	Per cent of total	Specific activity†	Total activity‡	Per cent of total	Specific activity§
Homogenate	2.64 (2.24)	100	0.82 (0.70)	0.77	100	0.25
N _w	0.080 (0.041)	3 (2)	0.16 (0.10)	0.096	12	0.17
M _{w2}	0.32 (0.29)	12 (13)	0.40 (0.35)	0.38	49	0.58
P _w	0.024 (0.012)	0.9 (0.5)	0.031 (0.024)	0.28	36	0.49
S ₂	2.17 (2.00)	82 (89)	1.82 (1.48)	0.057	7	0.053

The figures in parentheses represent an experiment in which 0.88 M sucrose was used as the medium for fractionation. All other figures are average values obtained in three experiments in which 0.25 M sucrose was used as the medium.

* Micromoles of TPN reduced per minute by 100 mg. of fresh liver or an equivalent amount of each fraction.

† Micromoles of TPN reduced per minute per mg. of total nitrogen.

‡ Micromoles of cytochrome c reduced per minute by 100 mg. of fresh liver or an equivalent amount of each fraction.

§ Micromoles of cytochrome c reduced per minute per mg. of total nitrogen.

genates was recovered in the final supernatant (S₂), and this was the only fraction in which the enzyme was concentrated in terms of total nitrogen. The amount of activity present in fractions N_w and P_w was insignificant, whereas the mitochondrial fraction (M_{w2}) contained an average of 12 per cent of the total.

The finding of a small but significant amount of isocitric dehydrogenase activity in the mitochondrial fraction presented some difficulty in the interpretation of the data. The possibility that the enzyme was origi-

nally associated with intracellular mitochondria but was lost from these particles during the fractionation procedure was considered, and an attempt was made to test this explanation by carrying out the fractionation in 0.88 M sucrose. Since, in this medium, the mitochondria retain their normal elongated shape (3), whereas in 0.25 M sucrose the mitochondria are all spherical, it seemed possible that the mitochondrial membranes had suffered some damage in 0.25 M sucrose. There was, however, no significant change in the distribution of the enzyme when 0.88 M sucrose was used (Table I). It was concluded from the data in Table I that mitochondria probably contain little isocitric dehydrogenase and that the small amount of enzyme found in the mitochondrial fraction may possibly have been adsorbed on the particles. The enzyme thus appears to be almost entirely localized in the soluble fraction of the cytoplasm of the liver cell.

Distribution of TPN-Cytochrome c Reductase—Most of the TPN-cytochrome c reductase activity of the liver homogenate was recovered in two particulate fractions, mitochondria (M_w) and submicroscopic particles (P_w) (Table I). Furthermore, the enzyme was concentrated in terms of total nitrogen to a considerable degree in both fractions. Although 12 per cent was recovered in the nuclear fraction (N_w), most, if not all, of this activity could probably be accounted for by the fact that the nuclear fraction contained unbroken liver cells and some free mitochondria. It should also be mentioned that the small amount of activity remaining in the final supernatant could have resulted, at least in part, from incomplete sedimentation of submicroscopic particles.

Oxidation of d-Isocitrate by Liver Fractions—Recently, Kennedy and Lehninger (6) have presented results which they interpret as indicating that the oxidation of substrates of the Krebs tricarboxylic acid cycle by rat liver is localized in mitochondria. In the presence of citrate, α -ketoglutarate, or of a mixture of pyruvate and oxalacetate, isolated mitochondria were found to take up oxygen at a much higher rate than either of two other fractions, nuclei and a supernatant containing submicroscopic particles and soluble substances. Data showing the rate of oxidation of the substrates by the original homogenates were not included, however, and it is therefore not possible to determine either to what extent, if any, these oxidative reactions were concentrated in mitochondria or what proportion of the original activity of the whole tissue was recovered in the three fractions.

In view of previous investigations indicating that cytochrome oxidase is almost exclusively a function of mitochondria (1, 2, 4), it is believed that in experiments involving such complex oxidative reactions recovery data are essential in order to avoid misleading results. Thus, in a study

of the distribution of any system ultimately dependent on cytochrome oxidase for oxygen uptake, the only fraction that could be expected to take up oxygen would be the mitochondria. The data of Table II, showing the results of a typical experiment in which isocitrate was added to liver fractions as an oxidizable substrate, illustrate the point. Of the total activity of the original homogenate, only approximately 30 per cent was recovered in the four fractions, and the only fractions showing appreciable uptake of oxygen were the mitochondria and nuclei, the latter probably because of its mitochondrial content. When all the fractions were recombined in the same proportion as was originally present in the homogenate, complete recovery of activity was obtained. Furthermore,

TABLE II
Oxidation of d-Isocitrate by Mouse Liver Fractions

Preparation	Total oxygen uptake ^a	Per cent of total	Q_{O_2} †
Homogenate.....	1070	100	330
Nw.....	70	7	100
Mw ₂	250	23	370
Pw.....	<10	<1	<10
S ₂	15 Ca.	1 Ca.	10 Ca.
" + Nw.....	160	15	
" + Mw ₂	390	36	
" + Pw.....	240	22	
" + Mw ₂ + Pw.....	760	71	
" + " + " + Nw.....	1070	100	

Measurements of O_2 uptake were made by using aliquots of the fractions equivalent to the aliquot of the homogenate.

^a Microliters of O_2 per hour per 100 mg. of fresh liver or an equivalent amount of each fraction.

† Microliters of O_2 per hour per mg. of total nitrogen.

it seems likely that the method of determination was largely a measure of TPN-cytochrome *c* reductase activity. This would be indicated by the fact that the specific isocitric dehydrogenase activity of liver is much greater than the specific TPN-cytochrome *c* reductase activity (Table I) and by the fact that the Fraction P_w, which contained a considerable amount of TPN-cytochrome *c* reductase but very little isocitric dehydrogenase (Table I) or cytochrome oxidase (4), provided marked stimulation of oxygen uptake when added to M_{w2} and S₂. The somewhat higher recovery of oxidative activity (Table II) than of isocitric dehydrogenase (Table I) in mitochondria can also be explained in this way, since TPN-cytochrome *c* reductase was found to be concentrated in mitochondria (Table I).

It should also be mentioned that a situation somewhat similar to the oxidation of isocitrate by liver fractions (Table II) was found by Schneider and Potter (5) using a mixture of pyruvate and oxalacetate as substrate.

DISCUSSION

Intracellular Distribution of Isocitric Dehydrogenase and TPN-Cytochrome c Reductase—As is shown by the data of Table I, isocitric dehydrogenase is apparently localized in the soluble fraction of the cytoplasm of the liver cell. It therefore appears that the Krebs cycle reactions are not exclusively a function of mitochondria, even though this cellular component, as discussed recently by Schneider and Potter (5), plays an important rôle in at least several phases of the cycle.

The distribution of TPN-cytochrome *c* reductase among the liver fractions was strikingly different from that of isocitric dehydrogenase. Although it can be obtained as a soluble protein (15), TPN-cytochrome *c* reductase was almost entirely associated with particulate components of the cell, being concentrated in mitochondria to a slightly greater extent than in submicroscopic particles. The finding of TPN-cytochrome *c* reductase activity in mitochondria confirms results reported by Horecker (15). It is of interest that DPN³-cytochrome *c* reductase, an enzyme having a function very similar to that of TPN-cytochrome *c* reductase, is concentrated to a much greater extent in the submicroscopic particles of both rat (16) and C₃H mouse (17) liver than in mitochondria. It also may be mentioned that, under the conditions of the methods of assay, the specific DPN-cytochrome *c* reductase activity of C₃H mouse liver (17) is approximately 9 times higher than the specific TPN-cytochrome *c* reductase activity (Table I). The relatively low activity of the latter enzyme suggests that it may be a limiting factor in the Krebs cycle reactions.

Remarks on Interpretation of Data Obtained by Cell Fractionation Technique—Past experience with methods of cell fractionation and the obvious possibilities for the production of artifacts during the procedure have led to the firm opinion on our part that a biochemical property can be ascribed to a given structural component of the cell only when at least three important criteria have been satisfied: (1) the cell fraction must be cytologically defined in terms of the intracellular component it represents,⁴

³ Diphosphopyridine nucleotide.

⁴ Green (18) has recently stated that the "cyclophorase complex" appears to be associated with mitochondria. This statement hardly seems justified, since both the method of preparation of "cyclophorase" and actual desoxypentose nucleic acid determinations (5, 19) would indicate that it contains most of the nuclear material of the homogenate as well as mitochondria.

(2) the validity of the method of assay for the biochemical property must be established, and (3) the biochemical property must be concentrated to a greater (preferably, considerably greater) extent in the cell fraction than in the original whole tissue. The first two criteria are self-explanatory; the third arises from a consideration of the possibility of adsorption of substances on cellular particles and of an overlapping of fractions that is not readily detectable by cytological means. Thus the presence of a relatively small proportion of the total enzyme activity in a single fraction, *e.g.* the isocitric dehydrogenase activity recovered in mitochondria (Table I), cannot at the present time be accepted as a positive cytochemical finding. In this respect, as demonstrated by the data of Table II, it is believed that results are liable to be misleading when complete recovery data are not available and when the method of enzyme assay is not a direct measure of the activity of the enzyme in question.

Another difficult problem associated with the use of the cell fractionation technique arises from the fact that when the cell membrane is disrupted the structural components of the cell are released into abnormal surroundings. Since cytological studies have demonstrated that at least two cellular components, nuclei (20) and mitochondria (3, 21, 22), possess well defined membranes, it appears entirely possible that these membranes may be so damaged in the process of cell disruption as to allow the escape of soluble substances. Although direct evidence demonstrating the state of integrity of the membranes is not at hand, it has been shown that a large proportion of the total nitrogen of mitochondria isolated in 0.25 M sucrose represents soluble proteins that are released when the mitochondrial membranes are disrupted and that can be characterized in the analytical ultracentrifuge (23).

As was shown in a previous publication (3), the choice of a medium is of considerable importance. It was demonstrated that the use of electrolytes caused aggregation of mitochondria and resulted in great difficulty in the separation by centrifugal fractionation of nuclei and mitochondria. More recently it has been found that the addition of KCl at a final concentration of 0.1 M to suspensions of submicroscopic particles in 0.88 M sucrose causes these particles to aggregate so that the clumps are readily visible in the light microscope and are of the same order of size as mitochondria. This observation suggests that the use of electrolytes also results in difficulty in the separation of mitochondria and submicroscopic particles.

Within the past year, a comparative study has been made of two media in which aggregation of particles does not occur, 0.88 M (hypertonic) and 0.25 M (isotonic) sucrose. Although 0.88 M sucrose possesses the advantage of preserving the normal morphological characteristics of mito-

chondria (3), its use makes necessary both the employment of very high centrifugal forces (9) in the fractionation procedure and the addition of relatively large amounts of sucrose to reaction mixtures. Some enzyme reactions are apparently inhibited by high concentrations of sucrose (24). These disadvantages are largely overcome by the use of 0.25 M sucrose, and present results indicate that there are no essential differences in the two media as far as the distribution of total nitrogen, cytochrome *c* (9), and a number of enzyme systems (4, 17) is concerned. For practical purposes, it would therefore appear that 0.25 M sucrose is at present the medium of choice.

SUMMARY

A study is reported of the distribution of isocitric dehydrogenase and TPN-cytochrome *c* reductase among fractions isolated by differential centrifugation from homogenates of C₃H mouse liver.

Over 80 per cent of the total isocitric dehydrogenase activity of the homogenates was recovered in a fraction containing the soluble material of the cytoplasm of the liver cell. Of the particulate fractions, nuclei and submicroscopic particles showed very little activity, whereas mitochondria contained approximately 12 per cent of the total. Results obtained with hypertonic (0.88 M) sucrose as the medium were essentially the same as those obtained with isotonic (0.25 M) sucrose.

Of the total TPN-cytochrome *c* reductase activity of the homogenates, over 90 per cent was recovered in the particulate fractions, 49 per cent being present in mitochondria and 36 per cent in submicroscopic particles. Under the conditions of the methods of assay, the specific TPN-cytochrome *c* reductase activity of mouse liver was found to be much lower than values previously reported for DPN-cytochrome *c* reductase.

Some of the difficulties involved in the interpretation of data obtained with the cell fractionation technique are presented, and it is concluded that several definite conditions must be fulfilled before a biochemical property can be ascribed to a given structural component of the cell.

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STUDIES ON UREA FORMATION IN SURVIVING LIVER SLICES*

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The now classical work of Krebs and Henseleit (1, 2) which led in 1932 to the formulation of the ornithine cycle has more recently been supplemented by observations which indicate that glutamic and aspartic acids are obligatory factors in the formation of urea (3-8). The purpose of this paper is to describe some experiments with rat liver slices in which isotopic substrates have been employed in an effort to gain further insight of the urea-synthesizing mechanism.

EXPERIMENTAL

Liver Slice System—Balance experiments (1, 9) have demonstrated that liver slices produce urea rapidly only when ammonia and CO₂ are present. That ammonia may be extensively involved in this reaction with liver slices is shown in Experiment 17 of Table III.

In our experiments liver slices were incubated with various amino acids and ammonia and, after an arbitrary period, urea and ammonia were isolated. Under the conditions described, the system we have used was able, in the presence of ornithine, to effect a 30 to 40 per cent conversion of ammonia nitrogen to urea in a period of 50 minutes.

We have tested the effectiveness of the preliminary washing in the removal of preformed urea and ammonium ions from the slices by the isotope dilution method: 30 mg. (0.5 mm) of isotopic urea containing 15.1 atom per cent excess N¹⁶ were added to 15 gm. (wet weight) of washed rat liver slices suspended in physiological buffer solution. A sample of urea isolated after 1 minute as the xanthidryl derivative was undiluted. After continued aerobic incubation at pH 7.35 and 38° for 50 minutes in the presence of no other substrate but glucose, the isotope content of the urea recovered (14.8 atom per cent excess) indicated that only 0.00068 mm of urea per gm. of wet slices had been formed. In a similar experiment with

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isotopic ammonium chloride, similar washing failed to eliminate all the ammonium ions from the slices. In Table I are presented some results obtained with slices washed by the standard method, in which the initial ammonia determination by isotope dilution was followed by aerobic incubation (with glucose) for 90 minutes. A comparable experiment in which enough cyanide was added to inhibit the cytochrome-linked reactions completely is also included. In both experiments 0.006 m.eq. of ammonium ion per gm. of wet liver slices was present in the washed material. The additional quantity of ammonium ion generated during the incubation must have arisen mainly as a result of autolysis. The differences between the increases observed in the normal and the poisoned slices may be as-

TABLE I
Ammonia Production in Liver Slices

Each flask contained 150 mg. of D-glucose; 0.50 mM of N^{15} ammonium chloride (containing 3.08 atom per cent excess N^{15}); 75 ml. of Krebs' bicarbonate buffer; liver slices, 15 gm. wet weight; pH 7.35. In Experiment 2, KCN was added to a final concentration of 0.02 M. Incubation under 95 per cent oxygen-5 per cent carbon dioxide.

Experiment No.	Time	N^{15} concentration of NH_3 N	NH_3 calculated
	min.	atom per cent excess	m.eq.
1	0	2.63	0.585*
	15	2.63	0.585
	30	2.54	0.606
	60	2.46	0.626
	90	2.35	0.656
2	0	2.60	0.592†
	90	2.00	0.770

* Found by direct determination 0.588 m.eq.

† Found by direct determination 0.592 m.eq.

cribed to the fact that in Experiment 2 all reactions in which ammonia is utilized (*e.g.*, urea formation, interaction with α -keto acid) were blocked. Actually, the presence of ammonium ions in the slices was of no disadvantage, as in more of the experiments ammonium chloride was added along with other substrates.

Ornithine—It was to be expected that, in addition to its catalytic rôle in the Krebs-Henseleit mechanism, ornithine may undergo oxidative deamination in the liver and almost certainly participate in the glutamic acid-proline cycle (10, 11). These reactions result in the liberation of ammonia. We have evaluated the over-all contributions made by these processes in our system by incubating liver slices with ornithine labeled with N^{15} in

either the α or the δ position. Each set of experiments included one in which normal ammonia and one in which no ammonia was added.

The results of these experiments are given in Table II. Incubation of α -N¹⁵-L-ornithine in either the presence or the absence of added ammonia

TABLE II

Utilization of Ornithine and Citrulline for Urea Formation

Each flask contained 100 mg. of D-glucose; substrates (including NH₄Cl) added to 0.010 M final concentration; 50 ml. of Krebs' bicarbonate buffer; liver slices, 10 gm. wet weight; pH 7.35. Incubation, 50 minutes under 95 per cent oxygen-5 per cent carbon dioxide, at 38°. N¹⁵ concentration of α -N¹⁵-L-ornithine monohydrochloride, 2.84 atom per cent excess; N¹⁵ concentration of δ -N¹⁵-L-ornithine monohydrochloride, 31.4 atom per cent excess; N¹⁵ concentration of carbamyl-N¹⁵-L-citrulline, 6.53 atom per cent excess.

Experiment No.	Substrates	Ammonia N recovered after incubation				Urea N recovered after incubation				Total recovery of N ¹⁵
		N ¹⁵ concentration	N ¹⁵ concentration of marked atom added	M.eq. N ¹⁵ based on (II)	Recovery of N ¹⁵ added	N ¹⁵ concentration	N ¹⁵ concentration of marked atom added	M.eq. N ¹⁵ based on (II)	Recovery of N ¹⁵ added	
		(I)	(II)			(I)	(II)			
		atom per cent excess	per cent		per cent	atom per cent excess	per cent		per cent	
3	α -N ¹⁵ -L-Ornithine	2.31	81.5	0.067	13.4	0.443	15.6	0.017	3.44	16.8
4	α -N ¹⁵ -L-Ornithine + NH ₄ Cl	0.506	17.8	0.071	14.3	0.115	4.05	0.012	2.34	16.6
5	δ -N ¹⁵ -L-Ornithine	1.29	4.11	0.0011	0.218	1.82	5.80	0.0095	1.91	2.13
6	δ -N ¹⁵ -L-Ornithine + NH ₄ Cl	0.427	1.36	0.0051	1.01	0.653	2.08	0.0065	1.31	2.32
7*	Carbamyl-N ¹⁵ -L-citrulline + NH ₄ Cl	0.016	0.25	0.0012	0.12	2.99	45.8	0.547	54.7	54.8

* This experiment was performed with 2 times the quantities used in Experiments 3 to 6.

(see Experiments 3 and 4 of Table II) resulted in the appearance of about 14 per cent of the α -amino nitrogen in the ammonia fraction and about 3 per cent in the urea. The oxidative process deaminated about 17 per cent of the ornithine in 50 minutes. Since the isotope concentrations found in the urea in both experiments were considerably lower than those in the

ammonia, the nitrogen of this amino group was not directly involved in the urea synthesis.

The data obtained from experiments carried out with δ -N¹⁵-L-ornithine (Experiments 5 and 6 of Table II) show that a total of only 2 per cent of the nitrogen of the δ -amino group of ornithine appeared in the ammonia and the urea fractions.

These data are of interest in view of the assertion by Bach (12-14) that a mechanism exists in liver slices for the production of urea directly from citrulline by an oxidative process.

The use of ornithine labeled with N¹⁵ at the δ position is equivalent to the use of the correspondingly labeled citrulline. In Experiment 6 (Table II) the isotope concentration of the ammonia rose from 0 to 1.36 per cent (of the isotope concentration of the labeled N atom added) at the end of the incubation. It is reasonable to assume that the relative average isotope concentration in the free ammonia was about half that of the final concentration ($1.36/2 = 0.68$ per cent) and certainly not greater. However, the relative average isotope concentration of the urea formed during the entire incubation period was 2.08 per cent, or almost 3 times the average assumed for the free ammonia. Similar considerations apply to Experiment 5 (Table II). This suggests that a small amount of urea can be formed from the δ -amino nitrogen of ornithine without prior conversion to ammonia. However, it could be argued that in such a heterogeneous system the labeled ammonia arising from the oxidation of the ornithine is produced only within the cells, in which case the isotope concentration of the free ammonia in the cells would be higher than that of the ammonia outside. Unless the diffusion of the ammonia in and out of the cells is rapid compared to the rate of formation of ammonia from the δ -amino groups of ornithine, the difference in the isotope concentrations of the ammonia in the two phases could become large. In this event the urea (even though formed from ammonia of the cells) could have a higher isotope concentration than the ammonia of the extracellular medium. The data of Experiment 4 do not support this argument; since the production of labeled ammonia was about 14 times as great as in Experiment 6, the urea formed had a lower isotope concentration than the free ammonia of the extracellular medium. Diffusion of ammonia must have been sufficiently rapid in this case to equalize practically the isotope concentrations of the ammonia in the two phases. The same must have been the case in Experiment 3. The evidence of Experiments 5 and 6 is therefore consistent with the view that oxidative hydrolysis of citrulline is only a small source of urea in the surviving liver slice.

Further evidence that the rôle of ornithine in this system is primarily catalytic comes from Experiment 9 (Table III). 0.5 mM of ornithine was

added to the liver slice system. During the 50 minute incubation period 0.317 m.eq. of urea nitrogen was formed. It was possible to recover 80 per cent of the added ornithine, about 20 per cent of it being destroyed during the incubation (Table II).

These experiments show that the formation of urea and the conversion of ornithine to other products are independent processes.

Citrulline—In terms of the ornithine cycle citrulline is represented as being the intermediary step in a sequence of two consecutive reactions.

Further evidence is supplied (Experiment 7, Table II) with regard to the central rôle played by citrulline in this sequence, by the use of N^{15} -carbamyl-labeled citrulline in the liver slice system. In agreement with predictions made from the ornithine cycle the N^{15} concentration of the urea comes close to 50 per cent of that of the isotopic carbamyl nitrogen of the added citrulline.

Glutamine—Leuthardt (15) has suggested that glutamine may be an obligatory intermediate in the synthesis of urea by liver slices. As only small quantities of urea are formed from glutamine in the absence of added ornithine, the quantitative significance of such a process is negligible. Moreover, as Krebs (9) has pointed out, it is not necessary to invoke the existence of a cycle specifically involving glutamine. We have, nevertheless, evaluated the significance of glutamine in our system with the help of amide-labeled glutamine.

In Table III are given some results of these experiments. Were glutamine an obligatory intermediate in either of the synthetic steps of the cycle, the urea should have had an isotope concentration of 50 per cent of that of the amide group of the glutamine. This was not the case. Not more than 8 per cent of the glutamine was hydrolyzed and the isotope concentrations in the urea in Experiments 8 and 9, in which citrulline and ornithine respectively were added, were but little greater than the estimated averages (half the final values) for the ammonia. Very little direct utilization of the amide group of glutamine could have taken place.

Amino Acids—The important position occupied in hepatic amino acid metabolism by glutamic and aspartic acids and the suggested relationship of these amino acids to the urea-forming process in isolated systems prompted us to attempt an estimation of their specific significance in the system with surviving liver slices.

To ascertain whether or not a specific participation of glutamic and aspartic acids could be demonstrated in the system with liver slices, an experiment (Table IV) of the dilution type with labeled ammonia, unlabeled amino acids, and ornithine was performed. The addition of the non-isotopic amino acids did not result in a greater dilution of the ammonia than was found in the control. This shows that little of the nitrogen of

the added amino acids merged with that of the ammonia and could suggest either that diffusion into the liver cells of these compounds was slow, or that the amino acids were not extensively deaminated, or both. However, in view of the increase in dilution of the N^{15} concentration of the urea nitrogen with increasing additions of aspartic acid, the relative constancy of the N^{15} concentration of the ammonia nitrogen cannot be ascribed to a

TABLE III
Utilization of Glutamine, Aspartic Acid, and Glycine Nitrogen for Urea Formation

Each flask contained 100 mg. of D-glucose; all substrates added to 0.010 M final concentration; 50 ml. of Krebs' bicarbonate buffer; liver slices, 10 gm. wet weight; pH 7.35. Incubation, 50 minutes under 95 per cent oxygen-5 per cent carbon dioxide, at 38°. N^{15} concentration of N^{15} -L-aspartic acid, 31.4 atom per cent excess; N^{15} concentration of N^{15} - NH_4Cl , 31.4 atom per cent excess; N^{15} concentration of amide- N^{15} -L-glutamine, 32.6 atom per cent excess.

Experiment No.	Substrate with N^{15}	Substrate without N^{15}	Ammonium N recovered after incubation		Urea N recovered after incubation	
			N^{15} concentration	N^{15} concentration of marked atom added	N^{15} concentration	N^{15} concentration of marked atom added
			atom per cent excess	per cent	atom per cent excess	per cent
8	L-Glutamine	L-Citrulline + NH_4Cl	1.30	3.99	0.760	2.33
9	"	L-Ornithine + NH_4Cl	1.25	3.84	0.782	2.40
16	L-Aspartic acid	L-Ornithine + NH_4Cl	0.224	0.713	0.939	2.99
17	NH_4Cl	L-Ornithine + L-aspartic acid	26.1	83.1	20.9	66.6
18	"	L-Citrulline + L-aspartic acid	26.3	83.8	16.4	52.2
19	L-Aspartic acid	L-Citrulline + NH_4Cl	0.166	0.529	0.691	2.20
20	Glycine	L-Citrulline + NH_4Cl	0.288	0.917	0.672	2.14

lack of penetration of the liver cell wall by the aspartic acid. This progressive dilution, taken together with the constancy of the isotope concentration of the free ammonia, is consistent with the view (6) that the nitrogen of aspartic acid may be directly involved in the urea-forming process. Experiment 13, in which equimolar concentrations of aspartic acid and labeled ammonia were used, suggests that almost half of the urea nitrogen was directly derived from the aspartic acid. The similar effects

of glutamic acid and alanine (Experiments 14 and 15, Table IV) might be expected in view of the presence of a vigorous transaminating system in liver.

In order to determine more directly the possible participation of aspartic acid, liver slices were incubated with ammonia, L-ornithine, and L-aspartic acid (Table III). As in these and other experiments (Table IV) in which the ammonia was labeled, the urea did not reach an isotopic concentration equal to the estimated average of that of the ammonia,¹ it appears that nitrogenous compounds other than ammonia may take part in the urea synthesis. That aspartic acid is one of these is shown by Experiment 16 (Table III).

TABLE IV
Relative Utilization of Ammonia and Dicarboxylic Amino Acids for Urea Formation

Each flask contained 100 mg. of D-glucose; N¹⁵-ammonium chloride (containing 3.01 atom per cent excess N¹⁵), and L-ornithine monohydrochloride to 0.010 M final concentration; 50 ml. of Krebs' bicarbonate buffer; liver slices, 10 gm. wet weight; pH 7.35. Incubation, 35 minutes under 95 per cent oxygen-5 per cent carbon dioxide, at 38°.

Experiment No.	Addition (final molarity)	N ¹⁵ concentration of ammonia N recovered after incubation	N ¹⁵ concentration of urea N recovered after incubation
		atom per cent excess	atom per cent excess
10		1.61	1.28
11	L-Aspartic acid, 0.002	1.71	0.999
12	" " 0.004	1.73	0.798
13	" " 0.010	1.53	0.686
14	L-Glutamic " 0.010	1.48	0.720
15	L-Alanine, 0.010	1.61	0.921

Further experiments were performed (Table III) to test whether a specific participation of dicarboxylic amino acid nitrogen is involved in the amination of citrulline. These likewise revealed the preferential utilization of ammonia by the liver slices. In Experiment 18, the average concentration of the ammonia must have been higher than the final concentration (84 per cent of that of the added ammonia). Since excess citrulline was present, the relative isotope concentration of the urea, had ammonia been the sole source of nitrogen in the amination, should have been about

¹ The data in Table I show that there is an immediate dilution by the normal ammonia present in the slices, followed by a gradual dilution due to liberation of ammonia from the slices and from the amino acids added. From data in Table I it is estimated that the relative average isotope concentration was close to 70 per cent of that of the added ammonia.

42 per cent. The fact that it was higher (52 per cent) can be explained by the recycling of the ornithine formed by the hydrolysis of the arginine. In this experiment but little of the aspartic acid yielded ammonia which could be used for the amination of the citrulline. A more sensitive test was made in Experiment 19, in which labeled aspartic acid was incubated with normal citrulline and liver slices. Again the relatively low isotope concentration of the ammonia at the end of the experiment shows that little oxidative deamination of the aspartic acid had occurred. If the average isotope concentration of the ammonia is estimated as half the final value and it is assumed that citrulline was aminated exclusively by ammonia, the urea could have had a maximal isotopic concentration of one-quarter of the final value observed for the ammonia. Actually, the isotope concentration of the urea was more than 16 times as great. This indicates a direct use of the amino group of the aspartic acid and constitutes further evidence for penetration of aspartic acid into the liver cell. This source of urea nitrogen is small, however, compared to ammonia. The addition of isotopic glycine also resulted (Experiment 20) in urea having a high isotope concentration, 9 times as great as if the citrulline had been aminated solely by ammonia. This last finding was unexpected and awaits elucidation.

Though in general the data of the experiments here reported seem to indicate that in the amination of citrulline ammonia is primarily involved, they are consistent with a contributory utilization of aspartate according to the cyclic scheme proposed by Ratner and Pappas (7). Through the kindness of Dr. Ratner we were able to perform isotopic experiments with the enzyme preparation prior to the publication of its description (6). These (Experiments 21 and 22, Table V) support their conclusion that in the isolated system the nitrogen for the amination of citrulline comes almost exclusively from the aspartic acid and not from ammonia. The addition of labeled glycine also results in urea containing some N^{15} which (Experiment 23) could not have been derived from ammonia. We must postulate either a slight direct utilization of glycine or a transamination from glycine to one of the keto acids usually considered part of the transaminase system. The present data are insufficient to distinguish between these two possibilities.

In Experiment 24 (Table V) in which equal amounts of labeled glutamic acid and of normal aspartic acid were added to the enzyme system, the isotope concentration in the urea was approximately half that obtained with labeled aspartic acid alone (Experiment 21). Since transaminase was presumably present in the preparation employed, the result could be ascribed not to a direct utilization but to a complete "equilibration" of the labeled nitrogen between the glutamic and aspartic acids in a period which must be short compared with the incubation time. Ketoglutaric acid or

oxalacetic acid would be required as a catalyst. The presence of labeled nitrogen in the free ammonia is evidence that some of the glutamic acid had been deaminated by oxidation.

Slices—Rats of the Sherman strain were killed by decapitation and exsanguinated; the livers were removed immediately and then stored in saline at 0°. Slices were cut with a Stadie (16) microtome and washed five times with 6 volume quantities of Krebs' bicarbonate buffer (17) at 0°, allowing 4 minute intervals with agitation to insure equilibration.

TABLE V

Urea Synthesis with Ratner-Pappas System

Each vessel contained potassium phosphate buffer, pH 7.5, 0.025 M; phosphoglycerate, 0.0125 M; adenosinetriphosphate, 0.00125 M; MgSO₄, 0.0033 M; other substrates, 0.005 M (all concentrations are final); final volume, 16 ml. Incubation, 40 minutes, at 38°. In all cases, 3.20 ml. of dialyzed beef liver acetone powder extract (not ethanol-fractionated) were added. The N¹⁵-L-aspartic acid had an isotope concentration of 31.4 atom per cent excess; the N¹⁵-L-glutamic acid hydrochloride, 3.15 atom per cent excess; the N¹⁵ glycine, 31.4 atom per cent excess; and the NH₄Cl, 31.4 atom per cent excess. Before addition solutions of these compounds were neutralized to pH 7.4.

Experiment No.	Substrates	Ammonia N recovered after incubation		Urea N recovered after incubation	
		N ¹⁵ concentration	N ¹⁵ concentration of marked atom added	N ¹⁵ concentration	N ¹⁵ concentration of marked atom added
		atom per cent excess	per cent	atom per cent excess	per cent
21	N ¹⁵ -L-Aspartic acid, NH ₄ Cl, L-citrulline	0.487	1.55	14.54	46.3
22	L-Aspartic acid, N ¹⁵ -ammonium chloride, L-citrulline	28.4	90.5	0.256	0.815
23	L-Aspartic acid, NH ₄ Cl, N ¹⁵ -glycine, L-citrulline	0.149	0.475	0.214	0.682
24	N ¹⁵ -L-Glutamic acid, L-aspartic acid, NH ₄ Cl, L-citrulline	0.107	3.40	0.674	21.4

The incubations were performed in large Krebs' reaction flasks (18) or round bottom flasks similarly equipped with inlet and outlet tubes for passing the 95 per cent oxygen-5 per cent carbon dioxide mixture through the tissue suspension.

Isolation of Ammonia and Urea Nitrogen—At the end of the designated time, the slices were removed from the supernatant liquid and washed with half the original volume of buffer, and the combined supernatants were centrifuged. In more of these experiments the supernatants were de-

proteinized at once by bringing them to 6 per cent concentration with trichloroacetic acid. In the experiments involving glutamine the activity of the slice system was first destroyed by the addition to the suspension of a few drops of saturated mercuric chloride solution, and a sample of ammonia nitrogen was obtained from the supernatant (after the centrifugation and washing) by permutit² before deproteinization with trichloroacetic acid.

The trichloroacetic acid was removed by continuous extraction with ether for 15 hours, and ammonia for N¹⁵ analysis was obtained from an aliquot by means of permutit. For the quantitative determination of the ammonium ion and amide ammonia liberated by the action of trichloroacetic acid, the solutions were brought to pH 11 with potassium carbonate (half saturated solution), and the ammonia was aerated into standard acid. Unless glutamine had previously been added, the values obtained with permutit were in good agreement with those obtained for the ammonia and amide nitrogen present by the aeration procedure.

The procedure used to isolate the urea nitrogen for N¹⁵ analysis was determined by the nature of the previous additions. In experiments involving ornithine, urea was isolated as the dioxanthidryl derivative (19), which after two recrystallizations from glacial acetic acid always showed satisfactory melting point and nitrogen content. Since xanthidrol is able to react with citrulline, in the experiments involving citrulline, solutions free of ammonia and amide nitrogen were treated with purified urease and a sample of the resulting ammonia was isolated with permutit.

In experiments in which quantitative determinations of urea nitrogen were made, this was done by removal of ammonia at pH 11 and incubation with urease at pH 7.

Isolation of Ornithine—Ornithine was isolated by ion-exchange methods from the tissue slice supernatants which had undergone the above treatment. The several fractions were combined and deproteinized with trichloroacetic acid. The insoluble residue of protein and excess xanthidrol was removed, and the supernatant extracted with ether for 24 hours. The solution was brought to pH 1 with hydrochloric acid and evaporated to dryness *in vacuo*. The crystalline residue was repeatedly extracted with hot absolute ethanol, the filtrate evaporated to dryness, and the residual syrup refluxed with 30 ml. of 6 N hydrochloric acid for 3 hours and taken to dryness. Hydrochloric acid and the dicarboxylic amino acids were removed on a column of Duolite-A4 in the free base form. The effluent was passed through a column of Amberlite IRC-50 previously treated with acetate at pH 7.0. The resin was washed with 250 ml. of water and the

² All the samples of permutit were washed ten times with water before liberation of ammonia with alkali.

ornithine was eluted with 0.1 N sodium hydroxide solution until the pH of the effluent showed a sharp change from 7.0 upwards, at which point qualitative tests with ninhydrin showed that the amino acid had been completely eluted as a sharply defined band. The eluate acidified with HCl was evaporated to dryness *in vacuo* and the residue benzoylated. The yield of ornithuric acid was 130 mg. (m.p.³ 186–188°), which represents 76 per cent recovery of the ornithine originally added to the system. The nitrogen from this sample had 0.003 atom per cent excess N¹⁵, demonstrating that no ornithine is synthesized in this system.

Materials

α -N¹⁵-L-Ornithine hydrochloride was synthesized by a modification of the procedure of Clutton, Schoenheimer, and Rittenberg (20). The α -bromo- δ -m-nitrobenzoylaminovaleric acid was esterified with diazomethane and aminated with N¹⁵-potassium phthalimide in the presence of CuO and KI. Yield, 57 per cent; N, 16.6; calculated, 16.7.⁴ The isotope concentration was 11.48 atom per cent excess N¹⁵.

Although this product gave a negative Nessler reaction, the hydrochloride was recrystallized in the presence of 5 per cent normal ammonium chloride in order to dilute any contaminating traces of isotopic ammonium salts.

Resolution of α -N¹⁵-DL-Ornithine—The DL-ornithine was resolved by the Sørensen procedure (21). In this, success was regularly attained only when the crystallization of the L salt of brucine was initiated by inoculation with brucine L-ornithurate monohydrate, m.p. 136°, prepared from pure L-ornithuric acid.

For resolution, a mixture of 6.00 gm. (35.6 mm) of normal DL-ornithine monohydrochloride and 0.845 gm. (5.0 mm) of α -N¹⁵-DL-ornithine monohydrochloride (containing 11.48 atom per cent excess N¹⁵) was benzoylated. 5 gm. (14.7 mm) of the resulting ornithuric acid were used for resolution. The yield of L-ornithine monohydrochloride was 0.664 gm. (54 per cent); N, 16.5; calculated, 16.6;⁴ N¹⁵, 1.416 atom per cent excess; $[\alpha]_D^{23} = +10.81^\circ$ ($c = 5.62$ in water). The Nessler reaction was negative.

δ -N¹⁵-L-Ornithine—Methyl coumalate, obtained from malic acid by von Pechmann's method (22), was converted to N¹⁵- α -pyridone according to Stetten and Schoenheimer (10). This was hydrogenated (10) and 5.20 gm. (52.3 mm) of the resulting N¹⁵- α -piperidone were converted to α -bromo- δ -N¹⁵-m-nitrobenzoylaminovaleric acid (20) which was aminated with a large excess of aqueous ammonia.

DL-Ornithine was isolated in an over-all yield of 38 per cent. A 2 per cent solution in water gave a negative response to Nessler's reagent.

³ All the melting points are uncorrected.

⁴ Corrected for N¹⁵ content.

The resolution was carried out as described above for the α -labeled ornithine. The δ - N^{15} -L-ornithuric acid melted at 187 – 188° and $[\alpha]_D^{20} = +9.95^\circ$ ($c = 10.0$ in water containing 1 equivalent of sodium hydroxide). Hydrolysis afforded δ - N^{15} -L-ornithine monohydrochloride. N, 16.7; calculated, 16.8;⁴ N^{15} , 15.7 atom per cent excess; $[\alpha]_D^{22} = +10.90^\circ$ ($c = 5.62$ in water).

N^{15} -Carbamyl-L-citrulline—The isotopic urea required for this preparation was made by the method of Bloch and Schoenheimer (23) from diphenyl carbonate and isotopic ammonia. M.p., 131° ; N^{15} , 6.53 atom per cent excess.

1.74 gm. (10.3 mm) of L-ornithine monohydrochloride were condensed with 3.00 gm. (50 mm) of this urea according to the procedure of Kurtz (24) and Gornall and Hunter (25).

Degradation of N^{15} -Carbamyl-L-citrulline—In order to confirm the presence of N^{15} in only the carbamyl group, a solution of 150 mg. (0.86 mm) of the above citrulline in 25 ml. of 0.11 M barium hydroxide solution was boiled for 8 hours under reflux in a slow stream of ammonia-free nitrogen. The ammonia, evolved in almost theoretical yield, was collected in standard sulfuric acid; N^{15} found, 6.51 atom per cent excess.

Ornithine was isolated from the hydrolysate as ornithine picrate and purified as ornithuric acid. Yield, 43 mg. (74 per cent). M.p., 186 – 188° . N, 8.20; calculated, 8.23; N^{15} , 0.002 atom per cent excess.

L-Ornithine Monohydrochloride—The L-ornithine monohydrochloride used in these experiments was prepared from L-arginine monohydrochloride by the enzymatic method of Hunter (26), with the arginase preparation described by Hunter and Dauphinee (27). N, 16.5; calculated, 16.6; $[\alpha]_D^{23} = +10.88^\circ$ ($c = 5.62$ in water). (Hunter (26) reports $[\alpha]_D^{22} = +10.98^\circ$ ($c = 5.60$ in water).)

L-Citrulline—A combination of the procedures described by Kurtz (24) and by Gornall and Hunter (25), with slight modifications, was employed to prepare this compound from L-ornithine monohydrochloride. N, 23.9; calculated, 24.0; $[\alpha]_D^{23} = +17.92^\circ$ ($c = 5.00$ with 1 eq. of HCl in water) (Gornall and Hunter report $[\alpha]_D^{22} = +17.9$ – 18.0° ($c = 5.00$ with 1 eq. of HCl in water).)

DL-Ornithine monohydrochloride was prepared from L-ornithine monohydrochloride by treatment with sodium acetate-acetic anhydride, according to the general procedure of du Vigneaud and Meyer (28). N, 16.5; calculated, 16.6. A solution of the hydrochloride in water, $c = 5.37$, showed no optical activity.

N^{15} -L-Aspartic Acid—We are indebted to Dr. Hsien Wu, who synthesized this compound in this laboratory (29), for providing us with a sample.

N^{15} -L-Glutamic Acid Hydrochloride—We are indebted to Mr. A. San

Pietro of this laboratory for this sample. It was prepared by crystallization of a mixture of N^{15} -DL-glutamic acid hydrochloride and L-glutamic acid hydrochloride, $[\alpha]_D^{22} = +31.4^\circ$, based on free glutamic acid ($c = 5.0$ in 2 N HCl). Isotope concentration, 3.15 atom per cent excess N^{15} .

N^{15} -Glycine—The procedure customary in this laboratory (30) was employed to prepare this compound.

N^{15} -L-Glutamine (*Amide-Labeled*)—This preparation was made from L-glutamic acid by the procedure of Bergmann, Zervas, and Salzmann (31).

DISCUSSION

In experiments with labeled ammonia and ornithine the isotope concentration of the urea formed was never equal to that of the ammonia (see Experiment 10, Table IV). This inequality cannot be due to utilization of normal ammonia formed by degradative reactions in the cell, for it would be necessary to assume that this normal ammonia is converted to urea before it can diffuse out. All experiments in which labeled ammonia was present showed at the end of the incubation period dilutions which demonstrated that diffusion of normal ammonia from the cells to the surrounding fluid had taken place. Further, the results of Experiment 4 clearly prove that equilibration of ammonia formed in the slice mixed with ammonia of the medium faster than it was converted to urea or any intermediate in the urea synthesis. These findings indicate that some non-isotopic nitrogenous compound present in the cell was also utilized in urea synthesis.

As suggested by Ratner and Pappas (7), glutamic acid and ammonia, on the one hand, and aspartic acid and glutamic acid, on the other, are "in equilibrium" through the von Euler *et al.* system (32) and the transaminase system respectively. Other amino acids can interact with these amino acids either directly or by the mediation of L-amino acid oxidase. Depending on the nature of the specific action ascribed to glutamic and aspartic acid, the transformations at either step of the ornithine cycle will be characterized by their dependence on reactions involving "equilibration" with ammonia.

Before an attempt is made to assay the significance of aspartic acid in the present work the question of diffusion of this amino acid into the liver slice must be considered. Experiments 10 to 13 (Table IV) offer evidence that aspartic acid enters the liver (slice) cell; as the concentration of non-isotopic aspartic acid is increased the isotope concentration of the urea decreases. This effect is also observed with glutamic acid and alanine. As we have already indicated, the amino acids exert their diluting effect on the isotope concentration of the urea not by serving as a source of normal ammonia, which would dilute the isotopic ammonia, but by some other

path. For example, in Experiments 13 and 15, in which normal aspartic acid and normal alanine were added, the isotope concentrations in the urea are 45 and 28 per cent lower respectively than the control (Experiment 10), though the isotope concentration in the ammonia is nearly the same in all three experiments. Further, when slices are incubated with labeled aspartic acid in the presence of normal ammonia (Experiments 16 and 19, Table III) the urea has an isotope concentration considerably higher than that in the ammonia. The data support the view that these three amino acids enter the cell at appreciable rates.

In the presence of labeled citrulline and normal ammonia the N^{15} concentration in the urea obtained is close to half the isotope concentration in the carbamyl group (Experiment 7, Table II). Conversely, when normal citrulline and labeled ammonia are added (Experiment 18, Table III) the urea formed has about half the concentration of the added ammonia. The presence of normal aspartic acid in the latter experiment could not have appreciably altered the isotope concentration in the urea, for, as is shown in Experiment 19, labeled aspartic acid in the presence of normal ammonia and citrulline produced a very low isotope concentration in the urea. These experiments indicate that in the conversion of citrulline to arginine ammonia is employed undiluted, whatever the mechanism involved. Such is not the case when ornithine and labeled ammonia are used (see Experiments 10 and 17). This suggests that the dilution observed is associated with the conversion of ornithine to citrulline.

If the system described by Ratner and Pappas were the main physiological pathway of arginine synthesis and urea formation, then ammonia as it enters the cell should rapidly be converted to glutamic acid which could aminate oxalacetic acid. This mechanism accords with our data only on the assumption that the cell contains no significant amount of either aspartic or glutamic acid, for these would immediately be available for urea synthesis and would dilute the isotope concentration of the urea. It is clear that there has been penetration of aspartic acid in the present experiments. Moreover, it is known that the concentrations of both aspartic and glutamic acids in liver slices are appreciable. Awapara (33) has found that liver contains about 0.5 mg. of free aspartic acid and 0.6 mg. of free glutamic acid per gm. of tissue. In these experiments, involving 10 gm. of liver tissue, and in which about 0.2 mm of urea was formed, these two amino acids could have supplied 0.08 mm of nitrogen for urea formation. The urea formed from normal citrulline should have had an N^{15} concentration of only 30 per cent of that of the ammonia, rather than the value of 50 per cent found. On the other hand, our data demonstrate that aspartic acid can be utilized for urea formation without prior conversion to ammonia. In Experiments 11, 12, and 13 the addition of normal aspartic

acid lowered the isotope concentration of the urea without diluting the labeled ammonia. When equimolar concentrations of aspartic acid and ammonia are added the isotope concentration in the urea drops to about half that found when no aspartic acid is added. Further, incubation with labeled aspartic acid results in urea having an isotope concentration higher than that found in the ammonia (Experiments 16 and 19, Table III). These data, while they confirm the direct utilization of the amino nitrogen of aspartic acid for urea formation in liver slices, do not indicate which reaction employs this nitrogen nor do they indicate that aspartic acid is an obligatory intermediate in the reaction mechanism.

Our experiments with glutamic acid, alanine, and glycine demonstrate that these amino acids also are capable of contributing nitrogen for urea formation without conversion to ammonia. These observations suggest that the actual process of urea formation in liver slices is the final resultant of several simultaneous processes associated with the operation of the ornithine cycle.

The differences between the results here reported and those of Ratner and Pappas are striking. Were the rate of diffusion of ammonia very much greater than that of the amino acids used in these experiments the mechanism proposed by Ratner and Pappas could explain much of our results. Such diffusion data are not available. However, other interpretations are possible. The disorganization of the cell may result not only in the loss of other more highly organized systems but also of regulatory mechanisms. Thus, for example, if in the liver cell citrulline were aminated by two amino acids under the influence of two different enzymes, in a manner analogous to the amination of ketoglutaric acid by either aspartic acid or alanine under the influence of two different transaminases, fractionation might separate one of these systems from the other. If the rate of formation of urea were determined by the concentration of citrulline, as is suggested by the work of Gornall and Hunter (34), then the single enzyme system could form as much urea as the two enzyme systems in the intact cell. It therefore seems that although the reactions studied by Ratner and Pappas undoubtedly proceed in the cell their quantitative significance *in vivo* remains to be completely evaluated.

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SUMMARY

1. An investigation with the aid of N^{15} of the ornithine cycle in the surviving rat liver slice system is reported.

2. N¹⁵- α - and δ -labeled L-ornithine and N¹⁵-carbamyl-labeled L-citrulline have been synthesized.

3. The use of α - and δ -labeled ornithine has demonstrated that in this system the function of this amino acid is almost exclusively catalytic. The amino groups contribute directly to urea formation to a negligibly small extent.

4. The use of N¹⁵-carbamyl-labeled citrulline provides further evidence that this amino acid is an intermediate in the conversion of ornithine to arginine.

5. Experiments with N¹⁵-amide-labeled glutamine have indicated that no specific function is attributable to the amide nitrogen of glutamine in the urea-forming process in this system.

6. It has been shown that in liver slices ammonia nitrogen is much more efficiently used for urea formation than the amino nitrogen of glutamic acid, aspartic acid, and alanine. However, the nitrogen for urea formation can, at least in part, be derived directly from the amino group of aspartic acid. Glutamic acid and glycine can contribute to this process.

7. Evidence is presented that aspartic acid and glutamic acid penetrate the liver cell in this system.

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THE BIOSYNTHESIS OF NUCLEIC ACID COMPONENTS STUDIED WITH C¹⁴

I. PURINES AND PYRIMIDINES IN THE RAT*

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Studies of the metabolism of purines and pyrimidines have been made in a variety of ways. Man, rats, pigeons, yeast, *Neurospora*, and bacteria have been used as experimental organisms. Edson, Krebs, and Model (2) have shown that ammonia and lactate stimulate the production of hypoxanthine by pigeon liver slices. Ammonia labeled with N¹⁵ was shown by Barnes and Schoenheimer (3) to be utilized *in vivo* in the formation of tissue purines and pyrimidines.

In studies on uric acid excreted by pigeons, it was found by Buchanan *et al.* (4-7) that carbon 4 of uric acid is derived from the carboxyl carbon of glycine, carbon 6 from carbon dioxide, carbons 2 and 8 from formic acid, and carbons 4 and 5 from the carboxyl and α -carbons of lactate. In these experiments the possible sources of each carbon atom of the uric acid molecule were demonstrated. These results were confirmed by Karlsson and Barker (8).

The amino group of glycine was found to contribute nitrogen 7 in uric acid of man (9), as well as in the nucleic acid purines of yeast (10) and of the rat (11). Greenberg (12) has demonstrated the incorporation of formate and carbon dioxide into hypoxanthine in pigeon liver homogenates.

The metabolism of birds is different from that of mammals in that birds form uric acid as the chief end-product of nitrogenous metabolism, while mammals form urea. In mammals the excretory product of the purines, allantoin in most animals and uric acid in man, constitutes a very small part of the total nitrogen excretion. The differences between the relative extent of purine metabolism in birds and mammals suggest the possibil-

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ity that the paths of metabolism and the precursors for purines may be different in these different animals.

Since the work to be discussed below was first described (1), Marsh (13) has reported that formate is a precursor of nucleic acid in both rats and pigeons, and MacLeod and Lardy (14) have shown that the incorporation of CO_2 into purines of rats is stimulated by biotin.

The biological synthesis of pyrimidines has been studied very little, owing in part to the difficulty of their isolation. Ammonia (3) and the nitrogen of glycine (15) and orotic acid (16) are reported to be incorporated into uracil; preformed uracil nitrogen is not (17). Other small molecules have been suggested by Mitchell and Houlihan (18) to be concerned with pyrimidine synthesis on the basis of experiments with *Neurospora*.

The present investigations concern the carbon precursors of tissue purines and pyrimidines in mammals, studied by administering to rats compounds labeled with radioactive carbon. The compounds tested as precursors were carbon dioxide, carboxyl-labeled acetate, carboxyl-labeled glycine, doubly labeled glycine, and formate. Guanine and adenine were isolated from tissue nucleic acids, and another fraction of adenine was obtained from purine derivatives soluble in cold trichloroacetic acid. Pyrimidines were isolated from the nucleic acids. Guanine and uracil were degraded to determine the location of some of the C^{14} in the molecules.

Methods

Animals—Growing rats of the Wistar strain were kept in wire metabolism cages and fed a ground stock diet during the experimental periods. Labeled compounds were mixed with the food or injected. Urine from all rats in each experiment was collected under toluene and pooled daily. The radioactivity of excreted urea was determined by removing free CO_2 from an aliquot of urine by aeration, then digesting with urease, and collecting the CO_2 in barium hydroxide. Respiratory CO_2 was collected by placing a rat in a glass container fitted with inlet and outlet tubes. After sweeping with CO_2 -free air for 10 to 15 minutes, a sample was collected by bubbling the air through dilute CO_2 -free NaOH , and precipitated with barium chloride.

Synthesis of Labeled Compounds—Sodium bicarbonate was made from barium carbonate containing C^{14} . Carboxyl-labeled acetate was made by the Grignard reaction, and carboxyl-labeled glycine from ethyl bromoacetate and potassium phthalimide, according to the modified procedure of Sakami, Evans, and Gurin (19). Doubly labeled glycine was synthesized from C^{14} -barium carbide by way of acetylene and acetaldehyde, which was oxidized to acetic acid with silver oxide and converted to glycine as described above (19). Formate was obtained by hydrolysis of hydrogen cyanide by the method of Kriebel and McNally (20).

Isolation of Compounds—Two fractions were isolated from the rat tissues: (a) "nucleotides," extracted with cold trichloroacetic acid, which yielded adenine after hydrolysis, and (b) mixed nucleic acids, extracted with sodium chloride, precipitated, and hydrolyzed to free the purines and pyrimidines.

Rats were killed by a blow on the head. The skin, feet, head, spine, and tail were rapidly removed and discarded; the gastrointestinal tract was removed, slit lengthwise, washed carefully, and treated with the rest of the carcass. Each carcass was passed through a meat grinder and then treated for 5 minutes in a Waring blender with 250 ml. of ice-cold 5 per cent trichloroacetic acid. The tissue residue was filtered, washed twice with cold trichloroacetic acid, and treated with lipide solvents as described below. The above operations were carried out in a cold room at 5°.

After adding 1/90 volume of concentrated H_2SO_4 , the trichloroacetic acid extract was boiled for 2 hours to hydrolyze the nucleotides and destroy the trichloroacetic acid. The solution was neutralized with 1:1 NaOH after partial cooling, and the calcium phosphate filtered and washed. Free purine was precipitated by the method of Krüger and Schmid with copper sulfate and sodium bisulfite (see Hitchings (21)). After removal of the copper with hydrogen sulfide in acid solution, the purine was reprecipitated with copper as before. The filtrate from a second treatment with hydrogen sulfide was concentrated to a few ml. by distillation *in vacuo*. When the solution was decolorized with charcoal and chilled, adenine hydrochloride crystallized. A second crop was obtained by concentrating the supernatant and adding about 2 volumes of alcohol. Both portions were combined and recrystallized until pure from 3 per cent hydrochloric acid. As pointed out by Kerr (22), this adenine is derived almost entirely from free and phosphorylated adenylic acid, other adenine nucleotides being present in very small amounts.

Lipides were removed from the trichloroacetic acid-extracted tissue residue by several extractions with boiling 3:1 alcohol-ether and ether.

Nucleic acids were extracted from the dry, lipide-free tissue in a 24 hour extraction with hot 10 per cent sodium chloride solution, as described by Barnes and Schoenheimer (3). The nucleates were precipitated with ethanol and washed with ethanol and ether. It was found that stickiness of the precipitate could be avoided by keeping the solution cold during centrifugation and omitting washing with dilute alcohol. The sodium nucleates were white and powdery, and gave a weak biuret test. Yields ranged from 3.9 to 5.1 per cent of the dry tissue weight. These nucleates were a mixture of pentose and desoxypentose types and were hydrolyzed without further purification.

The procedure for isolation of purines and pyrimidines was essentially that of Plentl and Schoenheimer (17). After hydrolysis of the nucleic acids

with hydrogen chloride in methanol, guanine was isolated as the sulfate, and adenine as the picrate. Pyrimidines were isolated after hydrolysis of the nucleotides in 20 per cent HCl at 180°. No appreciable amount of cytosine was isolated because of its conversion to uracil. A partial separation of the uracil-thymine mixture was accomplished by repeated fractional crystallization. The presence of thymine was confirmed by a strongly positive Woodhouse (23) diazo test on the pyrimidine solution of each experiment.

Degradation of Guanine—Two procedures were used: (a) hydrolysis with hydrochloric acid to glycine, in which the carboxyl and methylene carbons are derived from carbons 4 and 5, respectively, of guanine (24, 25), and (b) permanganate oxidation to carbon dioxide, urea, and guanidine.

Procedure A—Hydrolysis of about 100 mg. of guanine sulfate was carried out with concentrated HCl at 200°, according to the procedure of Wulff (26) as modified by Abrams, Hammarsten, and Shemin (10). In order to avoid dilution by non-radioactive carbon, the glycine was isolated as the copper salt rather than as an organic derivative. After hydrolysis the chloride and sulfate were removed, an excess of cupric hydroxide added, and the mixture heated in a boiling water bath for 10 to 15 minutes. The supernatant and washings were concentrated to a small volume, chilled, and absolute ethanol added to precipitate the copper glycine. The salt was recrystallized several times from small volumes of water. The crystals were blue needles containing 1 mole of water of crystallization.

The copper glycine was dissolved in approximately 10 ml. of water, acidified, and treated with hydrogen sulfide. One portion of the filtrate from copper sulfide was evaporated to dryness and oxidized to CO₂ with the Van Slyke-Folch solution. The radioactivity of this fraction represents the average concentration of isotope from carbons 4 and 5 of the original guanine. Another portion of the glycine filtrate from copper sulfide was aerated to remove H₂S, and decarboxylated with ninhydrin. The radioactivity of this BaCO₃ is due to carbon 4 of the original guanine.

Procedure B—Permanganate oxidation of guanine under the conditions described below gives rise to guanidine containing carbon 2, urea (mainly carbon 8), and carbon dioxide formed principally from carbons 4, 5, and 6. In a somewhat similar oxidation, Strecker (27) obtained guanidine, urea, oxaluric acid, and parabanic acid after treating guanine in HCl with KClO₄.

Guanine sulfate (30 to 60 mg.) was dissolved in a few ml. of CO₂-free water in a small flask fitted with a dropping funnel, an inlet tube extending to the bottom, and an outlet tube. 0.4 N (3 e) potassium permanganate was added to the flask in small portions as long as it was decolorized. The solution in the flask was aerated and the pH kept between 1 and 2. The

flask was first kept in cracked ice until completion of the slow reaction, then briefly at 100° with the addition of more permanganate and acid. The liberated CO₂ was collected but will not be reported (except in Table II), since it does not represent specific carbon atoms. After aeration was stopped, the solution was decolorized with a drop or two of dilute hydrogen peroxide, neutralized to pH 5, and the manganese dioxide removed by filtration and washed with hot water. Urea present in the filtrate was digested with urease in the presence of acetate buffer at pH 5. The CO₂ of this fraction was mainly but not entirely derived from carbon 8 of guanine.

Solid trichloroacetic acid was added to the solution containing urease and the protein filtered. An excess of silver nitrate was added, and silver guanidine precipitated by making alkaline with saturated barium hydroxide. The precipitate was centrifuged, washed, and decomposed with hydrogen sulfide. The filtrate was concentrated to a small volume *in vacuo*, and guanidine precipitated as the picrate. It was recrystallized until pure, as shown by the melting point. For low activity samples the picric acid was extracted with toluene and ether, and the colorless solution evaporated to dryness before oxidizing to CO₂.

The yields of urea by this procedure were 30 to 40 per cent of a mole per mole of guanine. Probably similar amounts of guanidine were formed because, in one experiment, 14 per cent of a molar equivalent was obtained as pure recrystallized guanidine picrate. Unless some rearrangement has occurred, this compound will contain only carbon 2 of guanine. Only small amounts of free urea and guanidine are oxidized by heating for 1 hour in a boiling water bath at pH 1 with an excess of permanganate (8 per cent for urea and 5 per cent for guanidine, with no guanidine converted to urea).

Degradation of Uracil—Uracil has often been oxidized to oxaluric acid (28–30). We have oxidized uracil with permanganate to carbon dioxide and oxaluric acid. The latter compound was hydrolyzed with alkali to urea and oxalic acid. The three products were obtained in yields of 90 per cent or higher. Oxaluric acid was isolated from a larger scale oxidation and identified as the anhydrous potassium salt (m.p. 224–225°, 16.4 per cent N), free acid (m.p. 205° with decomposition), and ethyl ester (m.p. 174° with decomposition).

The mechanism of this oxidation of uracil is not definitely known; therefore at present it is impossible to decide which carbon of uracil gives rise to CO₂ in the degradation. For this reason we shall report the average radioactivity of the three carbons, 4, 5, and 6.

20 to 30 mg. of uracil were dissolved in a few ml. of CO₂-free water in the aerating flask fitted with a dropping funnel. After sweeping with CO₂-free

air, a tube of saturated barium hydroxide was connected to collect the CO_2 liberated in the first step. Portions of 0.5 or 1 ml. of 0.4 N (3 e) potassium permanganate and a few drops of 0.5 N sulfuric acid were added at room temperature, the pH being kept at 6, as long as the permanganate was decolorized. 25 mg. of uracil required about 5 ml. of permanganate and 0.5 ml. of acid. After removing the barium hydroxide tube, any excess of permanganate was decolorized with a drop or two of dilute hydrogen peroxide, and the manganese dioxide filtered and washed with hot water. At this stage there was no free urea or oxalate in the solution. The solution was made strongly alkaline with 5 drops of 10 per cent sodium hydroxide and heated for 5 minutes in a boiling water bath. The tube was removed from the bath and neutralized to pH 6 to 6.5 with a few drops of 5 per cent acetic acid. 10 per cent calcium chloride was added dropwise until precipitation of the oxalate was complete, and the solution was neutralized to pH 7.5 with dilute ammonia. The calcium oxalate was centrifuged and washed thoroughly, then dissolved in 2 N sulfuric acid, and oxidized to CO_2 with permanganate. The supernatant and washings from the calcium oxalate contained urea. This was digested with urease in the usual way, and the CO_2 derived from carbon 2 of uracil was collected. The slight excess of calcium in this solution did not inactivate urease.

Thymine was a contaminant of the uracil isolated from animal tissues in these experiments. It was found that thymine, when oxidized under these conditions (kept at pH 6), gave about 40 per cent of the theoretical CO_2 for 1 equivalent of carbon. About the same fraction of urea was liberated after alkaline hydrolysis, but no oxalate was found.

The *purity* of each compound was determined by micro-Kjeldahl analysis. This is believed to be adequate, especially when combined with recrystallization to constant radioactivity.

Calculated nitrogen contents are as follows: guanine sulfate, $(\text{C}_5\text{H}_5\text{N}_5\text{O})_2 \cdot \text{H}_2\text{SO}_4$, 35.0 per cent; adenine hydrochloride, $\text{C}_5\text{H}_5\text{N}_5 \cdot \text{HCl}$, 40.8 per cent; adenine picrate, $\text{C}_5\text{H}_5\text{N}_5 \cdot \text{C}_6\text{H}_3\text{N}_3\text{O}_7$, 30.8 per cent; uracil, $\text{C}_4\text{H}_4\text{N}_2\text{O}_2$, 25.0 per cent; thymine, $\text{C}_5\text{H}_8\text{N}_2\text{O}_2$, 22.2 per cent.

Radioactivity measurements on samples were made after combustion by the wet oxidation mixture of Van Slyke and Folch (31), and collection of the CO_2 in saturated barium hydroxide solution. The barium carbonate was centrifuged, washed until free from alkali, and dried. An acetone slurry of the barium carbonate was evaporated slowly to form a uniform layer on metal disks 1 inch in diameter. Counting was done with a thin mica end window Geiger tube.

Observed counts were corrected by factors between 1.0 and 2.0 to a standard plate of "infinite thickness" (maximum self-absorption). With our equipment this is a plate containing 90 mg. of barium carbonate, or 5.47

mg. of carbon. All counts were then converted to counts per minute per mm of carbon. Samples were counted to a standard error of 5 per cent or less, except when the calculated values were low (*viz.* 10, 20). These latter values are given in Tables I through IV because it is believed that they represent traces of radioactivity, although the values may have a standard error of from 10 to 25 per cent. In each case in which sufficient compound was available, it was recrystallized to constant radioactivity. The majority of purine samples was checked in this way.

TABLE I
Administration of Labeled Bicarbonate

Sodium bicarbonate (350,000 c.p.m. per mm of carbon) in solution, 13 mg. per ml. or 54,000 counts per ml. Rat A received six injections, 1 ml. per hour; Rat B, six injections, 2 ml. per hour; Rat C, eleven injections, 1 ml. per hour; Rat D, four injections, 3 ml. per 3 hours. Total isotope 2.2×10^6 c.p.m. Tissues combined after nucleotide extraction.

	C.p.m. per mm carbon		C.p.m. per mm carbon
Nucleotide adenine, Rat A	60	Urinary urea	5,400
" " " B	150	Respiratory CO ₂	
" " " C	125	Rat A. 5-15 min. after injection	19,000
" " " D	135	" " 15-30 " " "	14,300
Nucleic acid		" " 30-45 " " "	9,800
Adenine	130	" " 45-60 " " "	7,900
Guanine	170	" B. 5-15 " " "	27,000
C-2 (guanidine)	10	" " 15-30 " " "	21,000
C-8 (urea)	0	" " 30-45 " " "	19,000
C-4, C-5 (glycine)	35	" " 45-60 " " "	13,600
C-6 (calculated)	770		
Uracil			
C-2 (urea)	650		
C-4, C-5, C-6	45		

Results

Labeled Carbon Dioxide—Four male rats, average starting weight 130 gm., were given intraperitoneal injections of isotonic sodium bicarbonate solution, as shown in Table I. Rats A, B, and C were sacrificed an hour after the last injections. Rat D was sacrificed 2 hours after the fourth injection.

Respiratory carbon dioxide was collected separately from Rats A and B in four portions during the 55 minutes preceding sacrifice. There was high radioactivity in the expired air. In the rat receiving injections of 1 ml. of bicarbonate, 27 per cent of the C¹⁴ in one injection was exhaled in

the 55 minute period. The rat receiving 2 ml. per injection exhaled 23 per cent in the same time.

The adenines of the nucleotide fractions were isolated as described and oxidized to CO₂ for isotope assay without analyses. The specific activities indicated that incorporation of labeled CO₂ varied with the total amount given, rather than with the size or the times of individual injections.

The tissues of all the rats were combined after nucleotide extraction. Bases isolated from the nucleic acids were recrystallized until the following analyses were obtained: guanine sulfate 34.8 per cent N, adenine picrate 30.7 per cent N, uracil 24.7 per cent N.

The two degradations of guanine showed most of the isotope to be in carbon 6. Of particular interest is the finding of a high concentration of

TABLE II

Administration of Carboxyl-Labeled Acetate

Sodium acetate (26,000 c.p.m. per mm of carbon) given to five rats by subcutaneous injection twice daily; 40 mg. per rat per day for 5 days. Total isotope 6.3×10^5 c.p.m.

	C.p.m. per mm carbon		C.p.m. per mm carbon
Nucleotide adenine	15	Urinary urea	
Nucleic acid		2nd day	130
Adenine	10	3rd "	210
Guanine		4th "	200
CO ₂	15	5th "	160
C-2 (guanidine)	0	Respiratory CO ₂	
C-8 (urea)	0	1.5 hrs. after first injection	320
Uracil		1.5 " " last "	75
C-2 (urea)	65	Before sacrificing	0
C-4, C-5, C-6	15		

C¹⁴ in carbon 2 of uracil. The specific activity of this carbon of uracil was similar to that calculated for carbon 6 of guanine. They both were much lower than the specific activity of the carbon of the mixed urinary urea. The bicarbonate carbon as indicated by the expired CO₂ had a still higher specific activity.

Carboxyl-Labeled Acetate (Table II)—Five rats, average starting weight 186 gm., were fed a powdered stock diet for 5 days while receiving subcutaneous injections of 20 mg. of labeled sodium acetate in 1 ml. of solution twice daily. The animals were sacrificed on the morning of the 6th day, about 16 hours after the last injection; average weight, 191 gm.

Respiratory CO₂ was collected from one rat at various times as shown in Table II.

Analyses of the compounds isolated are as follows: nucleotide adenine picrate 30.5 per cent N, nucleic acid adenine picrate 30.0 per cent N, guanine sulfate 34.5 per cent N, uracil 24.6 per cent N. Traces of radioactivity appeared in the whole purines but guanidine and urea from guanine showed no radioactivity.

Radioactivity was found in carbon 2 of uracil, probably owing to the incorporation of CO_2 . The radioactivity of the urea from the daily pooled urine was considerably higher than that of the carbon of the ureide group of uracil.

TABLE III
Administration of Carboxyl-Labeled Glycine

Glycine (10,700 c.p.m. per mm of carbon) fed to nine rats, 50 mg. per rat per day for 10 days. Total isotope 1.3×10^4 c.p.m.

	C.p.m. per mm carbon
Nucleotide adenine	300
Nucleic acid	
Adenine..	270
Guanine.	300
C-2 (guanidine) .	10
C-8 (urea)	10
C-4, C-5 (glycine) .	530
C-4 (glycine COOH)	1220
Uracil	
C-2 (urea)	10
C-4, C-5, C-6. . . .	0
Urinary urea.	10-20
Feces.....	10
Respiratory CO_2	10

Carboxyl-Labeled Glycine (Table III)—Nine rats, average starting weight 99 gm., were fed labeled glycine. The animals were sacrificed 12 hours after the last feeding, when the average weight was 128 gm.

The two samples of respiratory CO_2 had only a trace of radioactivity, as did the urea. The same was true for the ureide carbon of uracil. Not more than 1 per cent of the isotope was lost by way of the feces. There appears to be a high efficiency in metabolic utilization of the glycine when fed.

The tissues of all animals were pooled, and the purines and pyrimidines isolated by the usual methods. The analyses are as follows: nucleotide adenine picrate 30.6 per cent N, nucleic acid guanine sulfate 34.6 per cent N, adenine picrate 30.8 per cent N, uracil 24.8 per cent N.

Good incorporation of C^{14} into all the purines is evident. Glycine derived from guanine by treatment with HCl was found to have all of its

C¹⁴ in the carboxyl carbon. The data show that most of the isotope is in carbon 4 of guanine.

Doubly Labeled Glycine (Table IV)—Five male rats, average starting weight 110 gm., were fed the stock diet containing glycine labeled in both carbons. The animals were sacrificed on the morning of the 6th day, the average weight at this time being 123 gm.

Respiratory CO₂ was collected from one rat for 15 minute periods during the 1st, 2nd, and 5th days, and at 2 hour intervals for 24 hours during the 4th day. Little or no activity was found in these samples or in urinary urea.

Analyses of the compounds are as follows: nucleotide adenine hydrochloride 40.8 per cent N, nucleic acid guanine sulfate 34.9 per cent N,

TABLE IV
Administration of Doubly Labeled Glycine

Glycine (7900 c.p.m. per mm of carbon) fed to five rats, 18 mg. per rat per day for 5 days. Total isotope 9.5×10^4 c.p.m.

	C.p.m. per mm carbon
Nucleotide adenine.....	70
Nucleic acid	
Adenine.....	65
Guanine.....	75
C-2 (guanidine).....	0
C-8 (urea).....	20
C-4, C-5 (glycine).....	115
C-4 (glycine COOH).....	135
Uracil.....	10
Thymine.....	0
Urinary urea.....	0-10
Respiratory CO ₂	0-10

adenine picrate 30.8 per cent N, uracil 24.7 per cent N. Thymine was not isolated in large enough quantity to analyze chemically but was found to have no radioactivity.

The carbons of glycine were incorporated into the purines of nucleic acids and of the nucleotides in the rat. These purines had approximately the same C¹⁴ content, with nucleic acid guanine slightly higher than the others. The analyses of the degradation products of guanine indicated that the carbons of glycine were incorporated into positions 4 and 5 of the purine. It is probable that the specific activity of the glycine administered was too low to demonstrate the incorporation of the α -carbon into positions 2 and 8, as shown by Karlsson and Barker (8) in the pigeon.

Uracil contained practically no radioactivity. This is not in agreement

with Bergstrand *et al.* (15) who concluded that glycine was incorporated into the uracil ring when glycine labeled with N^{15} was fed.¹

Labeled Formate (Table V)—Five male rats, average starting weight 118 gm., were fed the stock diet including labeled sodium formate. The diet with formate was put in the cages each afternoon, and the animals were sacrificed on the morning of the 5th day.

A sample of respiratory CO_2 on the 4th afternoon had an activity of 50 c.p.m. per mm of carbon, and a sample before sacrificing on the 5th morning showed an activity of 230 counts. Urea in the urine of the last day contained 360 c.p.m. per mm of carbon. Formate appears not to enter the urea molecule in rats, except as it is oxidized to form CO_2 .

TABLE V
Administration of Labeled Formate

Sodium formate (173,000 c.p.m. per mm of carbon) fed to five rats, 50 mg. per rat per day for 4 days. Total isotope 2.5×10^6 c.p.m.

	C.p.m. per mm carbon		C.p.m. per mm carbon
Nucleotide adenine	4,490	Urinary urea, last day	360
Nucleic acid		Feces	140
Adenine	4,550	Respiratory CO_2	
Guanine	5,760	Before last feeding	50
C-2 (guanidine)	16,400	After " "	230
C-8 (urea)	12,500		
C-4, C-5 (glycine)	0		
Uracil			
C-2 (urea)	190		
C-4, C-5, C-6	60		
Thymine	60		

Analyses of the compounds isolated are as follows: nucleotide adenine hydrochloride 40.5 per cent N, nucleic acid guanine sulfate 35.0 per cent N, adenine hydrochloride 40.8 per cent N. These three compounds were recrystallized several times after the above analyses were obtained, to check for constant radioactivity; uracil 24.8 per cent N, thymine 23.2 per cent N.

Formate is an excellent precursor of purines in the rat. It contributes largely to carbons 2 and 8, as shown by the permanganate degradation. Carbons 4 and 5, obtained as glycine in the HCl degradation, had no radioactivity.

Formate is apparently not a direct precursor of pyrimidines, since uracil

¹ A private communication from Professor Hammarsten states that he has already found that the α -carbon of glycine is not incorporated into the pyrimidines.

and thymine were very slightly active as compared with the purines. The highest concentration of isotope in uracil was in the ureide carbon, probably owing to incorporation of CO_2 . The specific activity of thymine was less than that of uracil. This is in agreement with the suggestion that the desoxyribonucleic acids turn over less readily than the ribonucleic acids (32).

DISCUSSION

Our experiments demonstrate the incorporation of the carbon of CO_2 , glycine, and formic acid into the purines of the nucleic acids and the adenine of the tissue nucleotides of rats. CO_2 contributes carbon mainly to position 6 and not to positions 2 and 8. The carboxyl and the α -carbons of glycine may enter, respectively, positions 4 and 5. Formic acid contributes carbon to positions 2 and 8. These results are in agreement with the observations of Buchanan *et al.* (6, 7), made on the uric acid excreted by pigeons. We have failed to find the incorporation of the carboxyl group of acetate in carbons 2 and 8 of the purine structure.² The total amount of C^{14} administered as acetate by us was small for such an active metabolite, even though it was given in amounts 6 times those used in the experiment with doubly labeled glycine.

Although metabolic "dilution" figures do not have great significance in experiments carried out under such widely varying conditions, they are given here for comparison. Ratios of the specific activity of the compound as administered to that of isolated guanine are as follows: bicarbonate 2070, carboxyl-labeled glycine 37, doubly labeled glycine 109, formate 29.

The ratios of the specific radioactivities of guanine and adenine are of considerable interest. The finding of Brown *et al.* (33) that labeled dietary adenine is incorporated into nucleic acids as adenine, and converted to guanine with about 40 per cent less isotope, has been interpreted to indicate that adenine is a precursor of guanine. In the present studies, and in the work of others (3, 10, 11, 14, 15), the use of labeled precursors having small molecules usually leads to a similar but somewhat greater incorporation of the isotope in guanine than in adenine. In our experiments, the ratios of specific radioactivities of guanine and adenine of the nucleic acids ranged from 1.09 to 1.33. This suggests that adenine is not the precursor of guanine in these experiments.

Little is known concerning the metabolism of the pyrimidines. In their catabolism in mammals, the nitrogen is converted into urea (34, 17), while the purines, with somewhat similar structure, are excreted mainly as al-

² In a personal communication, Buchanan has stated that he has obtained results with pigeons similar to ours with rats. The source of error of the earlier experiments has been shown to have been due to the presence of labeled formate in the synthetic labeled acetate.

lantoïn or uric acid. The experiment with labeled sodium bicarbonate proves that the carbon of CO_2 becomes the ureide carbon of uracil. The extent of incorporation of radioactive carbon appears to be about the same in position 2 of uracil as in position 6 of guanine. In both instances the incorporation of C^{14} depends upon the concentration of C^{14}O_2 in the body. This utilization of CO_2 in the biological synthesis of uracil constitutes another example of the fixation of CO_2 in compounds important in metabolism in the body. The carbon of glycine, formate, and acetate is not used directly in the synthesis of uracil.

After feeding labeled glycine, very small amounts of radioactivity appeared in the expired CO_2 . Apparently the glycine, when fed in small amounts, is incorporated into the body to a very large extent and is broken down very slowly. As little radioactive CO_2 was formed, there was little incorporation of C^{14} into carbon 6 of guanine, carbon 2 of uracil, or the carbon of urinary urea.

The finding of radioactivity in the expired CO_2 of the formate experiment indicates that formic acid is oxidized in the rat. This is in agreement with the work of Sakami (35). It was oxidized very slowly in the experiment of Buchanan on the pigeon. Nevertheless it is obvious that formate entered readily into metabolic reactions in the experiments of both investigators as well as in ours.

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SUMMARY

The biological synthesis of purines and pyrimidines in rats was studied by administering precursors labeled with radioactive carbon. Adenine, guanine, uracil, and thymine were isolated from tissue nucleic acids, and adenine from nucleotides. Guanine and uracil were degraded to determine the position of the isotope within the molecule.

Carbon dioxide was found to contribute to carbon 6 of guanine, glycine contributed its carboxyl carbon to carbon 4 and its α -carbon to carbon 5, and formate was incorporated into positions 2 and 8. While CO_2 was incorporated into position 6 of the purines, it entered at a similar rate into position 2 of uracil, *viz.* the ureide carbon. This constitutes a new reaction for the fixation of CO_2 in a compound necessary for the body.

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THE BIOSYNTHESIS OF RADIOACTIVE FATTY ACIDS AND CHOLESTEROL*

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The biological synthesis of long chain fatty acids from acetate by slices of rat liver tissue was demonstrated by Bloch, Borek, and Rittenberg (1) in 1946, and has been repeated by several investigators (2, 3). The effect of insulin on this incorporation was studied by Bloch and Kramer (2), who reported that, whereas insulin inhibited the incorporation of labeled acetate into fatty acids, the combination of insulin and non-labeled pyruvate resulted in an increased incorporation of the labeled acetate.

It was of interest to determine whether other short chain fatty acids, as well as acetic acid, might be utilized by liver slices for the synthesis of higher fatty acids. This report is accordingly concerned with the utilization of carboxyl and methyl- C^{14} -acetic acid, β - C^{14} -pyruvic acid, and carboxyl- C^{14} -butyric, hexanoic, and octanoic acids for this biosynthesis. The experiments reported here reveal some information concerning the mechanism by which these substrates are incorporated into fatty acids. A study of the effect of insulin upon this process has also been made.

EXPERIMENTAL

Organic Syntheses—Carboxyl- C^{14} -acetic, butyric, and hexanoic acids were synthesized via the Grignard reaction as described by Sakami, Evans, and Gurin (4). The carboxyl- C^{14} -octanoic acid was prepared by condensation of *n*-heptyl bromide with $NaC^{14}N$ and subsequent hydrolysis of the nitrile. The butyric acid was purified by fractional distillation at 163–164°. The hexanoic acid was fractionally distilled at 90–92° and 5 mm., and the octanoic acid at 108–109° and 3 mm. Methyl- C^{14} -sodium acetate was purchased from Tracerlab, Inc. The β - C^{14} -sodium pyruvate was synthesized from methyl- C^{14} -sodium acetate (5) and analyzed for purity by the procedure of Friedemann and Haugen (6).

Methods—Slices of liver tissue, from fed rats of the Wistar strain, were

* Aided by a grant from the American Cancer Society administered by the Committee on Growth of the National Research Council. Methyl- C^{14} -sodium acetate and C^{14} -sodium cyanide, used in these investigations, were obtained from Tracerlab, Inc., on allocation from the United States Atomic Energy Commission. Barium carbonate was obtained from the United States Atomic Energy Commission.

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incubated in a Warburg apparatus at a temperature of 38°. The capacity of the vessels was 250 ml.; the gas phase was 95 per cent O₂-5 per cent CO₂. Each vessel contained 15 ml. of Krebs-Ringer bicarbonate solution (7) with an initial pH of 7.4, 2.5 gm. of liver tissue slices, and 1 ml. of a solution containing 6 to 12.8 mg. of the short chain fatty acid substrate in the form of its sodium salt at pH 7.4. When insulin was employed, 1 mg. was dissolved in 1 ml. of water, the pH adjusted to 7.4, and the solution added to the incubating medium. To the control vessel was added 1 ml. of bicarbonate solution to compensate for volume change. We employed three preparations of amorphous insulin and one preparation of crystalline insulin, the latter containing a small amount of glycerol and phenol as preservatives; *viz.*, amorphous insulin lots W-1302 and T-2536 (courtesy of Eli Lilly and Company), electrophoretically homogeneous insulin (courtesy of Sharp and Dohme, Inc.), and iletin, U-20, lots 3073-404426 (Eli Lilly and Company). Unless otherwise mentioned, the incubation time was 3.5 hours.

Following incubation, the liver slices were saponified in alcoholic KOH according to the method described by Kaplan and Chaikoff (8) for the quantitative separation of fatty acids. Cholesterol is removed by this procedure. In those experiments in which cholesterol was recovered, it was precipitated as the digitonide from alcohol and washed according to the modification of Srere, Chaikoff, and Dauben (9).

In order to remove any unutilized radioactive substrate, the saponified fatty acids were subjected to precipitation by the copper-lime procedure described by Lehninger and Smith (10). Fatty acids whose chain lengths are longer than 10 carbon atoms are found in the copper-lime precipitate, whereas all those of shorter length remain in the supernatant. The precipitate was routinely washed three times with water, acidified, then reprecipitated with alkali, and washed three additional times. The copper-lime precipitate was treated with 6 N HCl and the long chain fatty acids (*i.e.*, containing more than 10 carbon atoms) were extracted with petroleum ether. The petroleum ether extract was washed with 0.1 N HCl, and the solvent evaporated to dryness. The recovered fatty acids were weighed and analyzed for radioactivity. That this procedure effectively freed the recovered fatty acids from all contaminating substrate was determined by control experiments in which radioactive acetate was mixed with an artificial mixture of oleic, palmitic, and stearic acids. The recovered long chain fatty acids were non-radioactive. Similarly, incubation of liver slices or homogenates of liver with carboxyl-labeled octanoate in Krebs phosphate or Lehninger phosphate medium yielded, after fractionation by the above procedure, long chain fatty acids which contained no detectable radioactivity.

Radioactivity measurements were made with a thin mica window Geiger

tube employing BaCO_3 plates. The fatty acids were oxidized by the method of Van Slyke and Folch (11), and the evolved CO_2 was washed with a small amount of distilled water and trapped in saturated $\text{Ba}(\text{OH})_2$ solution. The BaCO_3 was mounted on a suitable plate as described elsewhere (12) and measured. The actual counts (above background) were corrected to the number of counts given by a plate of infinite thickness (13) and calculated as counts per minute per mg. of carbon. The radioactivities reported are subject to a standard error in counting of no greater than 5 per cent.

Results

It was found that Krebs-Ringer bicarbonate solution and the intact liver cell are necessary for long chain fatty acid synthesis from octanoate. No radioactivity was detected in the recovered fatty acids when phosphate buffer was employed with slices and radioactive octanoate. Similarly, homogenized liver tissue fails to convert labeled acetate or octanoate to long chain fatty acids in the presence of either phosphate or Krebs-Ringer bicarbonate media.

Experiments with Acetate—Both carboxyl and methyl-labeled sodium acetate were readily incorporated into long chain fatty acids by slices of liver from rats of the Wistar strain (Table I). The neutral equivalent of the recovered fatty acids consistently gave values of 290 ± 2 , which indicated an average chain length of 18 carbon atoms. In six consecutive experiments in which pooled liver slices were employed, we recovered 5.7 to 35 per cent of the administered radioactivity in the isolated long chain fatty acids. In the corresponding experiments with slices from the same pool, the addition of amorphous insulin to the incubation medium invariably produced an increase in the incorporation of acetate; the radioactivity recovered ranged from 11 to 50 per cent of the total radioactivity administered.

In general our experiments confirm the observation of Bloch, Borek, and Rittenberg (1) that the amount of acetate incorporated into long chain fatty acids is greater in liver slices from young rats than from older animals. Although the percentage of substrate incorporated in our experiments appears to be much greater than that reported by previous investigators (1-3), the concentration of substrate we employed (0.004 M or less) was very much lower than the lowest concentration (0.05 M) employed by Bloch (14). The total amount incorporated is of the same order of magnitude.

The acetate experiments cited above were performed with amorphous insulin. One experiment with a commercial preparation of insulin (letin, Eli Lilly and Company) showed no significant effect on the incorporation of substrate into fatty acids.

We could not confirm the finding of other investigators (2) that insulin

TABLE I

Conversion of Carboxyl or Methyl-Labeled Acetate and Pyruvate to Fatty Acids; Effect of Insulin upon This Process

Experiment No.*	Substrate	Insulin†	Radioactivity administered		Radioactivity recovered in fatty acids			Substrate incorporated‡
			C.p.m. per mg. C (A)	Total counts	C.p.m. per mg. C (B)		(B)/(A)	
						per cent	per cent	μM
5	CH ₃ C ¹⁴ OONa	—	5100	17,900	28	13.7	0.55	17.4
	"	+	5100	17,900	62	30.1	1.22	38.2
6	"	—	5100	17,900	12	5.7	0.24	7.4
	"	+	5100	17,900	20	11.3	0.39	12.5
7	C ¹⁴ H ₃ COONa	—	9600	33,800	41	7.8	0.43	11.0
	"	+	9600	33,800	64	12.5	0.67	17.2
8	"	—	9600	21,100	39	13.2	0.41	10.5
	"	+	9600	21,100	64	22.2	0.67	17.3
9	"	—	3200	5,600	21	9.0	0.66	21
	"	+	3200	5,600	30	14.1	0.94	30
10§	"	—	9600	16,900	70	35	0.73	23
	"	+	9600	16,900	111	50	1.16	37
11	CH ₃ C ¹⁴ OONa	+	5100	36,800	57	15	1.12	15.9
	" + CH ₃ COCOONa	+	5100	36,800	42	10	0.82	11.3
12	C ¹⁴ H ₃ COONa	+	9600	16,900	38	21	0.40	10.2
	" + CH ₃ COCOONa¶	+	9600	16,900	32	17	0.33	8.7
13	C ¹⁴ H ₃ COCOONa	—	3850	8,080	3.7	3.2	0.09	2.0
	"	+	3850	8,080	3.7	3.4	0.09	2.1
	C ¹⁴ H ₃ COONa	+	9600	16,900	29	14.1	0.30	9.6
14	C ¹⁴ H ₃ COCOONa	—	3850	8,080	3.7	5.0	0.09	2.0
	"	+	3850	8,080	3.9	5.1	0.10	2.1

* All the experiments designated by the same number were performed on aliquots of pooled liver slices.

† 1 mg. of amorphous insulin was employed per flask except where specified.

‡ Expressed per 100 mg. of recovered fatty acids and calculated as follows:

$$\frac{\text{Total recovered radioactivity (c.p.m.)}}{\text{c.p.m. per } \mu\text{M substrate}} \times \frac{100}{\text{mg. fatty acids}}$$

For example, in Experiment 5 (without insulin) 115 mg. of fatty acids were recovered. The factor 0.76 (carbon content of stearic acid = 76 per cent) was employed to calculate the carbon content; the acetate used contained an initial activity of 122 c.p.m. per μM.

$$\frac{115 \text{ mg.} \times 0.76 \times 28 \text{ c.p.m. per mg. C}}{122 \text{ c.p.m. per } \mu\text{M}} \times \frac{100}{115 \text{ mg.}} = 17.4$$

§ Electrophoretically homogeneous insulin; courtesy of Sharp and Dohme, Inc.

|| Final concentration of pyruvate 0.003 M.

¶ Final concentration of pyruvate 0.0015 M.

in the presence of non-labeled pyruvate stimulated incorporation of labeled acetate (Table I, Experiments 11 and 12). It should be observed, how-

ever, that we used relatively low concentrations of pyruvate in these experiments.

Experiments with β -C¹⁴-Pyruvate—Although it was possible to demonstrate the utilization of sodium pyruvate for the biological synthesis of long chain fatty acids, the addition of insulin produced little or no increase in the incorporation of pyruvate carbon into fatty acids (Table I, Experiments 13 and 14). The actual incorporation of pyruvate was quite low when compared with acetate.

Experiments with Carboxyl-C¹⁴-Butyrate, Hexanoate, and Octanoate—It is apparent (Table II) that all three of these substances can furnish carbon atoms for the synthesis of long chain fatty acids. The addition of amorphous insulin produced no consistent stimulatory effect on the incorporation of butyrate, hexanoate, or octanoate into fatty acids. In some experiments, the addition of insulin produced an increased incorporation of octanoate into long chain fatty acids, although this effect seemed to be quite variable. The authors have no explanation for the variability observed. In experiments on pooled slices 10.9 μ M of butyrate and 29.4 μ M of acetate (Experiment 18), 3.3 μ M of hexanoate and 8.8 μ M of acetate (Experiment 21), and 11.1 μ M of octanoate and 49.5 μ M of acetate (Experiment 28) were incorporated per 100 mg. of recovered fatty acids.

In several experiments the recovered saturated fatty acids were separated from the unsaturated by means of the lead salts as described by Twichell (15). Our results are in accord with previous reports (14, 16–18) that the saturated fatty acids contain much more labeled carbon than the unsaturated; e.g., the radioactivity of the biologically synthesized mixed fatty acids (Experiment 25) was 7.7 c.p.m. per mg. of carbon, while that of the saturated fatty acids was 12.5 c.p.m. per mg. of C.

Degradation of Biologically Synthesized Fatty Acids—Decarboxylation of the fatty acids was accomplished by modifying the method of Easterfield and Taylor (19). The fatty acid and pure iron wire were placed in an evacuated, sealed tube after preliminary flushing with nitrogen prior to evacuation. The fatty acid was heated at 300° for 1 hour; the liberated CO₂ was frozen with liquid nitrogen and subsequently released and trapped as BaCO₃ which was analyzed for radioactivity. With this method, we were able in control experiments to recover consistently 85 per cent of the specific activity of the carboxyl carbon of synthetic carboxyl-labeled octanoic acid (Table III). Biologically synthesized long chain saturated fatty acids recovered from experiments in which carboxyl-C¹⁴-acetate and octanoate were used as substrates were similarly decarboxylated (Table III). In all instances, it was observed that the specific activity of the carboxyl carbon was nearly twice the specific activity of the average carbon of the whole molecule. It is probable, therefore, that the resulting long chain fatty acids are labeled in alternating carbon atoms and that

TABLE II

Conversion of Carboxyl-Labeled Butyrate, Hexanoate, and Octanoate to Long Chain Fatty Acids

Experiment No.*	Substrate	Insulin†	Radioactivity administered		Radioactivity recovered in fatty acids			Substrate incorporated‡
			C.p.m. per mg. C (A)	Total counts	C.p.m. per mg. C (B)		(B)/(A)	
						per cent	per cent	μM
15	CH ₃ (CH ₂) ₂ C ¹⁴ OONa	—	2010	22,000	29	9.3	1.44	22.5
	"	+	2010	22,000	21	6.0	1.05	16.2
16	"	—	2010	11,000	16	10.4	0.80	12.4
	"	+	2010	11,000	17	10.0	0.85	13.1
17†	"	—	2010	22,000	3.5	1.3	0.17	2.7
	"	+	2010	22,000	4.5	1.8	0.22	3.6
18	"	+	2010	22,000	14	4.3	0.69	10.9
	C ¹⁴ H ₅ COONa	+	9600	56,300	90	10.8	0.93	29.4
19	CH ₃ (CH ₂) ₄ C ¹⁴ OONa	—	3510	26,200	8.2	2.1	0.23	2.4
	"	+	3510	26,200	9.1	2.2	0.26	2.7
20†	"	—	3510	30,800	7.3	1.7	0.21	2.2
	"	+	3510	30,800	8.6	2.1	0.24	2.6
21	"	+	3510	43,800	10.9	1.9	0.31	3.3
	C ¹⁴ H ₇ COONa	+	9600	56,300	26.8	3.9	0.28	8.8
22†	CH ₃ (CH ₂) ₆ C ¹⁴ OONa	—	1450	12,050	5.3	2.6	0.37	2.9
	"	+	1450	12,050	18.0	8.6	1.24	9.8
23	"	—	1450	13,500	3.9	2.4	0.27	2.1
	"	+	1450	13,500	6.1	3.6	0.42	3.2
24†	"	—	1450	14,500	2.4	1.6	0.17	1.3
	"	+	1450	14,500	4.9	3.6	0.34	3.2
25	"	—	1450	5,800	7.7	4.9	0.53	4.2
	"	+	1450	5,800	6.5	4.3	0.45	3.6
26	"	—	1450	5,800	17.3	9.0	1.20	9.5
	"	+	1450	5,800	23.7	12.5	1.64	13.0
27	"	—	1450	5,800	7.0	4.2	0.48	3.8
	"	+	1450	5,800	8.0	4.7	0.55	4.4
	"	—	1450	5,800	7.0	3.9	0.48	3.8
	"	+	1450	5,800	8.0	4.5	0.55	4.4
28	"	+	1450	19,300	20.4	6.2	1.41	11.1
	C ¹⁴ H ₇ COONa	+	9600	56,300	150	15.2	1.57	49.5

* All experiments designated by the same number were performed on aliquots of pooled liver slices.

† 1 ml. of iletin was used per flask; 1 mg. of amorphous insulin per flask was used where not specified.

‡ Expressed per 100 mg. of recovered fatty acids. See the foot-note to Table I.

octanoate is not incorporated intact into long chain fatty acids to any major extent, but undergoes a preliminary cleavage into 2-carbon fragments which are subsequently utilized for the synthesis of fatty acids.

Cholesterol—All of the substrates investigated contribute carbon atoms for the synthesis of cholesterol by surviving liver slices (Table IV). In all cases, the cholesterol was analyzed as the digitonide.

TABLE III
Decarboxylation of Fatty Acids
Results in counts per minute per mg. of C.

Substance decarboxylated	Radioactivity of whole molecule	Radioactivity of carboxyl carbon	
		Found	Theory
Carboxyl- C^{14} -octanoic acid	100	675	800
Saturated long chain fatty acids derived from carboxyl-labeled acetate	34	54	68*
Saturated long chain fatty acids derived from carboxyl-labeled octanoate	20	33	40*
“ “	26	42	52*

* Calculated on the basis that the C^{14} is located in alternate carbon atoms of the fatty acid molecule.

TABLE IV
Biosynthesis of Cholesterol by Liver Slices

Experiment No.	Substrate	Radioactivity of substrate (A)	Radioactivity of recovered cholesterol* (B)	(B) (A)
		c.p.m. per mg. C	c.p.m. per mg. C	per cent
8	$C^{14}H_7COONa$	9600	520	5.4
13	$C^{14}H_7COCOONa$	3850	175	4.6
16	$CH_3(CH_2)_2C^{14}OONa$	2010	49	2.4
20	$CH_3(CH_2)_4C^{14}OONa$	3510	58	1.7
27	$CH_3(CH_2)_6C^{14}OONa$	1448	84	5.6

Calculated from the digitonide.

DISCUSSION

It is evident that acetic, pyruvic, butyric, hexanoic, and octanoic acids furnish carbon atoms for the synthesis of long chain fatty acids and cholesterol by liver slices obtained from rats of the Wistar strain. The failure of this conversion of octanoate in phosphate buffer is of interest, since Bloch (14) has observed a stimulating effect of oxalacetate upon the synthesis of fatty acids from acetate in this buffer medium. Whether bicarbonate buffer is superior to phosphate because of increased formation of oxalacetate (thus making available more energy) is a matter that needs further study. Bloch (14) has discussed some of these aspects.

The stimulating action of insulin upon the incorporation of acetate into

fatty acids was analyzed statistically and found to be highly significant ($P < 0.01$). The effect of insulin on the incorporation of the other substrates into long chain fatty acids was, in general, not so significant and in some instances quite variable. That the effect upon acetate incorporation is due to insulin and not to some contaminant is indicated by the similar action of electrophoretically homogeneous insulin.

Experiments with carboxyl-labeled acetic and octanoic acids as substrates indicate that the specific radioactivity of the carboxyl carbon of the biologically synthesized long chain fatty acids was almost twice that of the average carbon for the whole molecule. These findings are in keeping with the observations of Rittenberg and Bloch (18) with regard to the distribution of labeled carbon atoms in long chain fatty acids derived from C^{13} -labeled acetate. Our observations with octanoic acid may best be explained by proposing that the octanoic acid is, to a considerable extent, cleaved to 2-carbon fragments which are subsequently recondensed to form long chain fatty acids. As a result of this mechanism, radioactivity would be evenly distributed in alternating carbon atoms of the fatty acid molecule.

Reference to the final column of Table II indicates that 1 mole of octanoate is incorporated under conditions in which 4.5 moles of acetate are utilized. Similarly, 1 mole of hexanoate is incorporated while 2.7 moles of acetate are utilized. Although the correlation is not good in all cases, it would appear from these observations that the short chain fatty acids are almost as efficiently converted by liver slices to long chain fatty acids as is acetate.

SUMMARY

Liver slices from rats of the Wistar strain are capable of synthesizing long chain fatty acids and cholesterol from acetic, pyruvic, butyric, hexanoic, and octanoic acids. With this tissue, the conversion of octanoate to long chain fatty acids occurs readily in bicarbonate buffer but not in phosphate buffer.

The addition of insulin to the incubating medium markedly stimulates the incorporation of acetic acid into long chain fatty acids. No statistically significant effect of insulin was observed with pyruvic, butyric, hexanoic, and octanoic acids.

The long chain fatty acids derived from carboxyl-labeled octanoate appear to contain C^{14} in alternating carbon atoms throughout the molecule. These results suggest that the synthesis of long chain fatty acids from short chain acids probably occurs, to a large degree, by fragmentation of these acids to 2-carbon units which are subsequently recombined to form long chain fatty acids.

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THE COLORIMETRIC DETERMINATION OF UREA IN THE BLOOD OF NORMAL AND UREMIC RATS*

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In connection with other studies in this laboratory (1, 2),¹ it became necessary to determine the concentration of urea in the blood of individual new-born rats. In view of the small quantities of blood available and the need for a considerable number of analyses, the colorimetric method developed by Archibald (3) appeared to be particularly suitable. This method is based upon the formation of a colored compound when strongly acid solutions of urea and α -isonitrosopropiophenone are heated together. As applied to human blood, this procedure was reported by Archibald to yield results which corresponded closely to those determined with a manometric urease method. Inasmuch as some other compounds, such as allantoin, citrulline, alloxan, and even proteins if present in sufficient quantities, give rise to a color similar to that produced by urea (3), the specificity of the Archibald method when applied to rat blood was not assured. When it was found that under certain conditions the urea concentration in the blood of very young rats, as measured by this method, rose to extremely high levels, further studies on the specificity of this method were clearly indicated. Such studies might also reveal extensive variations in the concentration of chromogens other than urea in the blood of rats. In this paper data are reported on (a) a comparison of the colorimetric method and a urease method for determination of urea in rat blood and (b) the extent of destruction by urease of substances which are chromogenic in the colorimetric method.

EXPERIMENTAL

Colorimetric Determination of Urea—Blood was collected from decapitated rats directly into dry 0.2 ml. Kahn serological pipettes graduated into 0.001 ml. divisions. The measured sample was then discharged into 2 ml. of distilled water and the pipette was rinsed with the water. The proteins were precipitated with zinc hydroxide according to Somogyi (4). The resulting suspension was then diluted so that the final volume was

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¹ Halvorson, H. O., and Schultze, M. O., unpublished work.

from 40 to 80 times that of the blood used and centrifuged. Urea was then determined in duplicate on 1 ml. aliquots of the deproteinized diluted blood as outlined by Archibald (3), with use of a Coleman junior spectrophotometer for measurement of optical densities at 540 $m\mu$. Standard solutions prepared from recrystallized urea and containing from 10 to 50 γ of urea were run simultaneously. By close adherence to standardized procedure, the reproducibility of the method was very satisfactory. Thus, the mean optical density at 540 $m\mu$ of 62 external standards containing 10.7 γ of urea was 0.155 ± 0.0011 (standard error of the mean); the mean optical density of fifteen internal standards of the same concentration was 0.156 ± 0.002 . The optical density of the colored compound was directly proportional to the concentration of urea in the range of 10 to 50 γ of urea.

Urease Method for Determination of Urea—The procedure used was that of Kinsey and Robison (5) in which the ammonia liberated by the action of urease (The Arlington Chemical Company, Yonkers, New York) diffuses into a drop of glycerol saturated with boric acid and is then titrated with standard acid and bromocresol green as an indicator. A micro burette was constructed from a 1 ml. syringe; it held a volume of 438.2 c.mm. and the plunger was moved by an attached micrometer screw graduated into 1000 divisions. The mean recovery of ammonia from six aliquots of a standard ammonium chloride solution containing 55 γ of nitrogen per sample was 99.4 ± 0.22 per cent (standard error of the mean). When this procedure was tested with a standard solution of recrystallized urea, the mean recovery from seven aliquots containing 29.5 γ of urea was 100.05 ± 0.41 per cent (standard error of the mean). For determination of urea in pooled blood specimens by this procedure the final dilution of blood to reagents plus water was 1:4.7 or 1:15.1 with normal and uremic blood, respectively. To 275 c.mm. of deproteinized, diluted blood 90 c.mm. of a freshly prepared 0.75 per cent solution of urease in 0.10 M phosphate buffer of pH 7.0 were added and the digestion was carried out at room temperature for 45 minutes in the closed system. After addition of 90 c.mm. of saturated potassium carbonate to raise the pH to >12.0 the ammonia was allowed to diffuse into the boric acid for 2 hours at 37° and then titrated with 0.02 N sulfuric acid.

Table I shows the precision obtained when the colorimetric and urease methods were applied to several aliquots of pooled samples of deproteinized normal and uremic blood.

Destruction by Urease of Chromogens Reacting with α -Isonitrosopropiophenone—For these experiments a freshly prepared 0.75 per cent solution of urease in 0.1 M phosphate buffer, pH 7.0, was added to deproteinized blood with a final dilution of about 1:15. After 45 minutes at room

temperature the solutions were deproteinized again with zinc hydroxide and aliquots were treated with the reagents used for the determination of urea by the method of Archibald. The residual color was calculated in terms of urea, although it was produced by chromogens other than urea which react with α -isonitrosopropiophenone. This was ascertained by the observation that urea added to blood was destroyed completely by the quantity of urease used, even if urea were present in amounts exceeding 4 times that found in uremic blood. Blank determinations showed that the urease preparation contributed only traces of chromogenic material; these could not account for the color developed with α -isonitrosopropiophenone in many specimens which had been treated with urease.

TABLE I

Precision of Colorimetric and Urease Methods Applied to Pooled Samples of Normal and Uremic Blood

	No. of aliquots	Urea per 100 ml. whole blood	
		Urease method	Colorimetric method
		mg.	mg.
Normal rats	6	37.78 \pm 0.18*	37.41 \pm 0.41*†
Uremic "	7	190.0 \pm 1.21	211.0 \pm 1.69‡

* Standard error of the mean.

† This specimen of pooled blood from 21 day-old rats is one of the two found which failed to produce a color with α -isonitrosopropiophenone after treatment with urease.

‡ This specimen gave a residual color after urease treatment equivalent to 16 mg. of urea per 100 ml. of blood.

RESULTS AND DISCUSSION

The results of these comparative studies summarized in Table II establish that (a) the colorimetric method as applied to rat blood gives results which are about 12.5 per cent higher than those obtained by the urease method, (b) rat blood usually contains one or more chromogens other than urea which react with α -isonitrosopropiophenone (the quantity of these non-urea chromogens increases approximately in proportion to the increase of urea in the blood of uremic rats), (c) the condition of "acute uremia of the newborn" described elsewhere (6) is a true uremia. Since the amount of urease used in these experiments was sufficient to decompose quantities of urea far in excess of those present in the blood filtrates obtained from uremic rats, the residual color developed after urease treatment must be due to non-urea chromogens present in rat blood. We have observed this residual color in all of the thirteen specimens of uremic rat

blood and in eight out of ten specimens of normal blood. The intensity of the residual color in the latter specimens was quite weak (optical density 0.02 to 0.04) and hence little significance should be ascribed to the magnitude of these values. In the uremic bloods, however, the mean residual color was equivalent to 14.8 mg. of urea per 100 ml. of blood.

Of the various compounds which have been shown by Archibald (3) to give a color with α -isonitrosopropiophenone similar to that produced by urea, allantoin, citrulline, and traces of protein or polypeptides are those most likely to be present in the deproteinized filtrates from uremic blood. Allantoin has been reported to occur in the blood of rats older than those with which we have worked to the extent of 0.85 to 1.67 mg. per 100 ml. by Christman *et al.* (7) and 7.7 mg. per 100 ml. by Hawkins *et al.* (8). According to the data of Archibald (3), allantoin, under the conditions of the

TABLE II

Comparison of Colorimetric and Urease Methods of Analysis and Presence of Non-Urea Chromogens in Blood

	Urea per 100 ml. whole blood	
	Normal rats, 10 specimens	Uremic rats, 13 specimens
	mg.	mg.
(a) Colorimetric method	37.4 \pm 1.7*	208.2 \pm 11.2*
(b) Urease method	33.2 \pm 2.0	185.2 \pm 10.0
(c) Residual color after urease action, calculated as urea	2.6 \pm 1.1	14.8 \pm 1.9
(a - b)/b \times 100	12.7	12.4
c/b \times 100	7.8	8.0

* Standard error of the mean.

colorimetric method used here, gives an optical density of only about 7 per cent of that produced by an equal weight of urea. Even if it were assumed that the residual color observed in the samples obtained from uremic blood was due solely to allantoin, no valid estimate of its concentration in the blood could be made from the present data because it has been found that the urease preparation used reacts with allantoin. Thus when amounts of allantoin ranging from 0.24 to 1.7 mg. were treated with urease under the same conditions as were used for deproteinized blood and then deproteinized and treated with α -isonitrosopropiophenone, the allantoin treated with urease yielded only from 49 to 56 per cent of the color obtained with untreated allantoin. The presence of allantoinase and other enzyme activity in commercial preparations of urease has been pointed out by others (9, 10). The fact that the sum of the urea equivalent of the

residual color and the urea determined by the urease method does not equal the values obtained colorimetrically (Table II) may reflect the action of the urease preparation on allantoin in the blood. The urease preparation which was used for these experiments, however, did apparently not contain allantoicase, since no ammonia was evolved from allantoin by the enzyme. Therefore, the presence of allantoin in the blood did not affect the urea determinations made with the urease method as reported here.

The interference by citrulline in the colorimetric determination of urea is even much less than that of allantoin (3). No data appear to be available on the concentration of citrulline in rat blood. In uremic human blood Archibald found a citrulline concentration of not more than 2 mg. per 100 ml. If this amount of citrulline were present in rat blood, it would not be sufficient to cause an appreciable residual color in the colorimetric analyses reported here. De Verdier and Ågren (11) have reported that filtrates prepared with zinc hydroxide from the blood of cattle contain small amounts of protein. Tests for protein in the blood filtrates prepared in this study were negative. The presence of appreciable quantities of polypeptides in the blood filtrates is made unlikely by the observation that the filtrate prepared with zinc hydroxide from a tryptic digest of soy bean protein, when treated with α -isonitrosopropiophenone, formed a color which had an entirely different absorption spectrum from that of the residual color observed with urease-treated filtrates from uremic blood. The latter, with α -isonitrosopropiophenone, yield absorption curves similar to those given by low concentrations of allantoin or urea with a maximum at 530 m μ . The absorption maxima of the colored compound obtained when urea or blood filtrates from uremic rats are treated with α -isonitrosopropiophenone are at 540 m μ . While the chemical nature of the non-urea chromogens present in uremic rat blood requires further study, the evidence obtained is not inconsistent with the view that allantoin is involved to a large extent.

SUMMARY

1. A comparative study of the determination of urea in the blood of normal and uremic rats by the colorimetric method of Archibald and the urease method of Kinsey and Robison has shown that the former yields, in general, results which are about 12 per cent higher than those obtained with the latter.

2. The chemical nature of the compounds responsible for the high values obtained in the colorimetric method has not been identified.

3. The "acute uremia of the newborn rat" has been characterized as a true uremia.

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THE OXIDATION OF ACETATE*

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Intact animals, yeast, and bacteria can readily metabolize acetate, but isolated animal tissues possess limited activity toward this substrate (1, 2), the rate being most rapid with slices of guinea pig and rabbit kidney cortex (3, 4).

There is evidence that acetate is converted to carbon dioxide and water via the tricarboxylic acid cycle (5-7), but little is known concerning the initial steps in the activation of this substrate.

Attempts to determine the components of the enzyme system involved in the oxidation of acetate by employing cell-free enzyme preparations have generally met with little success. Distillers' yeast, treated with liquid nitrogen to destroy the membrane (8), and a lysed preparation of *Micrococcus lysodeikticus* (9) can oxidize acetate. Grafflin and Green (10) have reported a washed preparation of rabbit kidney cortex which oxidizes fatty acids and acetic acid and have demonstrated the "sparking" effect of α -ketoglutaric acid on acetate oxidation.

Inorganic phosphate and Mg^{++} or Mn^{++} were necessary for the condensation of acetate and oxalacetate to form citrate in kidney mitochondria (11). In an important report by Stern and Ochoa (12), adenosinetriphosphate, coenzyme A, and Mg^{++} catalyzed this reaction in an extract of pigeon liver acetone powder.

The present communication describes various optimum conditions necessary for acetate oxidation by a washed homogenate of rabbit kidney and the distribution of this system in the various fractions of the washed homogenate. With well washed preparations, inorganic phosphate, magnesium ions, adenosinetriphosphate, oxalacetic acid, or another of the 4-carbon

* The experimental data in this paper are taken from a dissertation submitted by W. B. Elliott in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa in February, 1950. This work was supported by a stipend from the Graduate College of the State University of Iowa, and by grants from the Nutrition Foundation, Inc., and Smith, Kline and French Laboratories, and was presented in part at the meeting of the American Society of Biological Chemists at Detroit, April 18-22, 1949.

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dicarboxylic acids from the tricarboxylic acid cycle, and pantothenate are necessary for activity.

The work on pantothenate and coenzyme A was greatly stimulated by the reports from Lipmann's laboratory and are a confirmation of the report of Stern and Ochoa. This paper is a study of factors of acetate oxidation in kidney, as compared to Stern and Ochoa's work on liver, the enzyme being investigated in the insoluble mitochondrial system rather than in the extracted soluble form.

Methods

Tissue Preparation—Rabbit kidney cortex (obtained immediately after the animal was killed) was homogenized in a Potter-Elvehjem glass homogenizer in 10 volumes of 0.9 per cent NaCl solution. The tissue suspension was washed by centrifuging at approximately $19,000 \times g$ for 5 minutes in the cold and resuspending the residue in fresh NaCl solution. The residue obtained after two or more washings, as indicated in each experiment, was suspended in 2 volumes of NaCl solution.

The activity of the preparations was measured in the standard Warburg manometric apparatus at 30.2° with various additions as indicated in each experiment. Acetate was tipped in from the side bulb after 10 minutes temperature equilibration. For determining oxygen uptake, 0.3 ml. of 10 per cent KOH with folded filter paper strips was placed in the alkali well. CO_2 evolution was measured by the "direct method" of Warburg, as described by Umbreit *et al.* (13). The data are corrected for blank O_2 and CO_2 values and are taken from experiments representative of the series performed on each factor.

Boiled kidney juice was obtained by homogenizing kidney cortex in 2 volumes of 0.9 per cent NaCl, boiling for 5 minutes, and then removing the precipitated proteins by filtration. Acetone powders of kidney cortex were prepared according to the method of Kaplan and Lipmann (14).

Fractionation of Cellular Components—The tissue, homogenized in 5 volumes of 0.9 per cent NaCl, was fractionated essentially as described by Claude (15, 16) and Schneider (17) to obtain a residue fraction (containing nuclei, whole cells, and debris), a mitochondrial fraction, and a supernatant, referred to as the soluble fraction plus microsomes. All centrifugations were carried out at approximately 3° . The precipitates were washed two or more times in 0.9 per cent NaCl solution and finally made up with 2 volumes of 0.9 per cent NaCl. In all cases the volumes of 0.9 per cent NaCl used were based on the original tissue weight.

Chemical Methods—Acetate was determined by a modification of the steam distillation method of Friedemann (18). The samples from the flasks were deproteinized with an equal volume of freshly prepared 10 per cent metaphosphoric acid and made up to a total volume of 10 ml. 5 ml. ali-

quots of the protein-free filtrates were steam-distilled after the addition of 3 gm. of dry MgSO_4 . The fluid volume in the distillation flask was held at 10 ml. and 10 volumes of distillate collected. The distillate was aerated for 10 to 15 minutes with CO_2 -free air and then titrated with 0.01 N NaOH. Blank and control determinations were made for each experiment. The amount of volatile acids in the flasks without added acetate was very small.

Succinate was determined by the method of Szent-Györgyi and Gozsy as improved by Krebs (13).

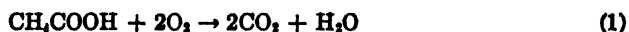
Citrate was determined by the method of Pucher *et al.* (19) with modifications as previously described (20).

Adenosinetriphosphate was isolated from rabbit skeletal muscle following magnesium anesthesia (21), according to the methods of Needham (22) and Szent-Györgyi (23). Oxalacetate was prepared according to Krampitz and Werkman (24) and was dissolved and neutralized immediately before use. Lithium pyruvate was prepared according to Wendel (25). Acetate, succinate, and fumarate were commercial products. Coenzyme A was prepared according to Kaplan and Lipmann (14), and purified samples of coenzyme A were graciously supplied by Dr. Fritz Lipmann and Parke, Davis and Company. Sodium pantothenate was obtained through the courtesy of Hoffmann-La Roche, Inc.

Results

Complete Oxidation of Acetate by Kidney Cortex Preparations

Kidney cortex washed once was capable of completely oxidizing acetate, as shown in Table I. The data in Table I indicate complete oxidation according to the following equation.



The activity of the enzyme was proportional to the enzyme concentration up to 1.0 ml. of the preparation. The pH optimum (in glycine buffer) was broad, with highest activity at pH 8.0 to 9.2; the activity fell off sharply above pH 9.2 and gradually below pH 8.0. Oxygen did not increase the rate of acetate oxidation over that in air. In preliminary experiments, various other rabbit tissues, such as liver, heart muscle, brain, spleen, lung, skeletal muscle, and diaphragm, were tested for their ability to oxidize acetate. Kidney cortex was most active; liver showed a slight activity.

Effect of Magnesium, Manganese, and Phosphate Ions on Acetate Oxidation

With kidney cortex homogenate washed twice, magnesium and inorganic phosphate ions were required for activity. Manganese would not replace magnesium on an equimolar basis but did so if used in lower concentrations

(Table II). Concentrations of magnesium lower than 0.005 M gave only slight increases in activity over the blank. The requirements of magnesium and phosphate ions for acetate oxidation are similar to those for citrate formation by a similar preparation (11).

TABLE I
Complete Oxidation of Acetate by Washed Kidney Cortex Homogenate

Reaction measured		Per cent of tl
	μM	
Acetate utilization....	13.1	
Oxygen uptake....	25.6	97.7
Carbon dioxide evolution....	26.7	102.0

Flask contents, once washed homogenate 0.8 ml., acetate 100 μM , Sørensen's glycine buffer, pH 8.2, 0.4 ml., Na_2HPO_4 0.01 M, MgCl_2 0.01 M, pantothenate 150 γ . Total volume, 2.0 ml. plus KOH in the center well or H_2SO_4 in the side arm as required. Time, 2 hours.

TABLE II
Effect of Various Ions on Acetate Oxidation

Added ions	O_2 uptake		
	Experiment 1	Experiment 2	Experiment 3
	μM	μM	μM
	0.98	0.71	
Mg^{++} (0.01 M)	8.10	5.09	4.82
Mn^{++} (0.01 ")	0.81	0.40	0.81
" (0.001 M)			4.35
" (0.0001 M)			5.40
PO_4^- (0.01 M)	10.15	7.90	
Mg^{++} , PO_4^- (0.01 M)	12.90	13.02	
Mn^{++} , " (0.01 ")	4.55	3.39	

Flask contents, once washed homogenate (Experiment 1), twice washed homogenate (Experiments 2 and 3), 0.8 ml.; glycine buffer, pH 8.2, 0.4 ml.; acetate 100 μM . Total volume, 2.0 ml. plus KOH in alkali well. Time, 2 hours (Experiments 1 and 3), 90 minutes (Experiment 2).

Effect of Adenosinetriphosphate and Members of Tricarboxylic Acid Cycle on Acetate Oxidation

The addition of boiled kidney juice to the twice washed homogenate gave a considerable increase in oxygen uptake and carbon dioxide evolution over the addition of magnesium, phosphate, and pantothenate. Therefore various additions were made in an effort to find the additional factors necessary. Adenosinetriphosphate was tried because of the re-

quirement for it in acetylation reactions (11, 26, 27). The necessity for adenosinetriphosphate is shown in Table III. Concentrations under 0.001 M were only slightly effective. Inorganic phosphate was necessary for oxidation, even in the presence of adenosinetriphosphate.

In view of the evidence in favor of the oxidation of acetate via the tricarboxylic acid cycle, various members of the cycle were added. All the members of the cycle tested (except pyruvate) caused increased activity

TABLE III
Effect of Adenosinetriphosphate on Acetate Oxidation

ons	Oxygen uptake
	μM
Complete system	29.4
No adenosinetriphosphate	14.0
" magnesium	2.3
" phosphate	3.0

Flask contents, twice washed homogenate 1.8 ml., adenosinetriphosphate 0.001 M; other additions (except glycine) as in Table I. Total volume, 3.0 ml.

TABLE IV
Effect of Various Members of Tricarboxylic Acid Cycle on Acetate Oxidation

Experiment No.	Addition	Oxygen uptake	Acetate utilized
		μM	μM
1		3.1	14
	Succinate (10 μM)	6.3	27
	Malate (5 μM)	6.0	21
	Fumarate (5 μM)	4.6	24
	Pyruvate (5 ")	0.7	
2		7.7	
	Oxalacetate (2 μM)	12.0	
3		0.5	
	Oxalacetate (1 μM)	3.4	

Flask contents, tissue in Experiments 1 and 2 four times washed residue fraction 1.6 ml., in Experiment 3 twice washed mitochondria 1 ml., adenosinetriphosphate 0.001 M; additions as shown. Other additions as in Table I. Total volume, 3.0 ml.

(Table IV). This was in agreement with the necessity of oxalacetate for citrate formation from acetate (11, 12). An induction period, similar to that shown by Lynen (28) in acetate oxidation by yeast, was observed in the absence of added oxalacetate (Fig. 1) and was eliminated by the addition of 2 μM of oxalacetate per flask. The induction period apparently represents the time necessary for the formation of oxalacetate from its precursors in sufficient quantity to prime the system.

Distribution of Acetate Oxidation System in Cell

The acetate oxidation system was present in approximately equal amounts in the residue fraction and the mitochondrial fraction. The ac-

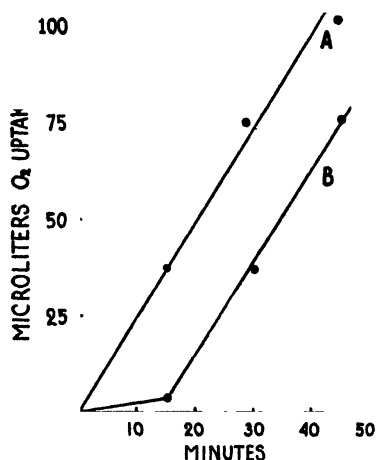


FIG. 1. The effect of oxalacetate on the induction period in acetate oxidation. Curve A, acetate (100 μ M) plus oxalacetate (2 μ M); Curve B, acetate alone. Flask contents, four times washed residue fraction 1.6 ml., adenosinetriphosphate 0.001 M, oxalacetate and acetate as indicated; other additions as in Table I. Total volume, 3.0 ml.

TABLE V
Oxidation of Acetate by Various Tissue Fractions

Fraction	Succinate added	Oxygen uptake
	μ M	μ M
Residue.....		7.4
".....	5	19.0
Mitochondria...		4.2
".....	5	13.4
Microsomes + soluble globulins		0.0
" + " "	5	0.9

Flask contents, tissue as indicated 1.0 ml., succinate as indicated; other additions as in Table III.

tivity of the microsome plus soluble globulin fraction on acetate was very slight (Table V).

Lability of Acetate Oxidation System

In the early part of the work on acetate oxidation, considerable difficulty was experienced owing to the rapid loss of activity (70 per cent or more) occurring when the tissue preparation was held at 0° for even short

periods of time before use. With the addition of adenosinetriphosphate, oxalacetate, and pantothenate to the flasks, the tissue preparation could be held for an hour with no loss in activity.

Effect of Pantothenate and Coenzyme A on Acetate Oxidation

In view of the effect of pantothenate on pyruvate oxidation (29-31), and the effect of coenzyme A on acetylation reactions (32-34), pantothenate

TABLE VI
Effect of Pantothenate on Acetate Oxidation

Na pantothenate	Oxygen uptake
γ per ml.	μM
	1.9
25	1.4
50	5.8
200	8.2

Flask contents, twice washed mitochondria 1.0 ml., pantothenate as indicated, adenosinetriphosphate 0.001 M, succinate 10 μM ; other additions as in Table I. Total volume, 3.0 ml.

TABLE VII
Effect of Coenzyme A on Acetate Oxidation

Tissue fraction	Coenzyme A, 100 units	Oxygen uptake	Increase due to coenzyme A
		μM	per cent
4 times washed residue, aged 1 hr.	—	75 (1 hr.)	
	+	95	27
4 " " " " 2 hrs.	—	54 (1 hr.)	
	+	72	33
Twice washed mitochondria, aged 8½ hrs.	—	202 (3 hrs.)	
	+	286	41

Flask contents, tissue preparation as indicated 1.0 ml., adenosinetriphosphate 0.001 M, oxalacetate 5 μM ; other additions (except pantothenate) as in Table I. Total volume, 3.0 ml.

was tested on acetate oxidation. With whole homogenates washed once or twice there was a small increase of oxygen uptake on the addition of pantothenate, and pantothenate was added routinely to the flasks. With twice washed mitochondria, added pantothenate caused a decided increase in oxygen uptake with acetate as a substrate (Table VI).

The addition of coenzyme A to similar tissue preparations or to dialyzed preparations gave no increase in oxygen uptake. If the tissue fractions were washed and aged, there was an increased oxygen uptake on the addition of coenzyme A (Table VII).

TABLE VIII

Effect of Coenzyme A on Citrate Formation by Extract of Acetone Powder of Rabbit Kidney Cortex

Additions	Citrate formed μM
Complete system	0.62, 0.83, 0.79
No acetate and oxalacetate	0.10
" coenzyme A	0.12
" enzyme	0.11

Tube contents, extract (2 gm. of acetone powder of rabbit kidney cortex extracted with 15 ml. of 0.02 M NaHCO_3) 0.3 ml., MgCl_2 0.01 M, phosphate buffer, pH 7.4, 0.01 M, adenosinetriphosphate 0.003 M, acetate 20 μM , oxalacetate 50 μM , coenzyme A 10 units. Total volume, 1.0 ml. Incubated at 37° for 1 hour; reaction stopped with 1.0 ml. of 10 per cent trichloroacetic acid. Duplicate samples were combined for analysis.

TABLE IX

Effect of Inhibitors on Acetate Oxidation

Inhibitors	Oxalacetate added μM	Oxygen uptake μM	Acetate utilized μM
	10	8.8	18
Arsenite (0.005 M)	10	0.3	16
" (0.01 M)	10	0.0	
	10	4.0	24
Iodoacetate (0.003 M)	10	0.0	15
		10.9	13
Malonate (0.005 M)		0.0	2
" (0.01 M)		0.0	0
" (0.005 M)	10	3.9	10
		9.8	
Fluoroacetate (0.003 M)		2.0	

Flask contents, four times washed residue 1.0 ml., inhibitors and oxalacetate as indicated; other additions as in Table III. Time, 1 hour.

TABLE X

Formation of Succinate from Acetate in Presence of Malonate

Malonate (0.005 M)	Acetate utilized μM	Oxygen taken up μM	Succinate formed μM
—	14	9.8	0
+	10	3.9	5.5

Flask contents, four times washed residue fraction 1.0 ml., malate 10 μM ; other additions as in Table III.

Coenzyme A is involved in citric acid synthesis by extracts of acetone-dried pigeon liver (11), yeast, and *Escherichia coli* (35)¹ and heart ventricle homogenates (36). An extract of acetone powder of rabbit kidney cortex also requires coenzyme A for citrate formation from acetate plus oxalacetate (Table VIII). Since most cellular pantothenate is present as coenzyme A (37), these results indicate involvement of pantothenate (as coenzyme A) in acetate activation for citric acid synthesis. However, other functions for pantothenate in acetate oxidation are not excluded.

Effect of Inhibitors on Acetate Oxidation

Arsenite, iodoacetate, fluoroacetate, and malonate all strongly inhibited the oxidation of acetate (Table IX). If oxalacetate was added, the oxygen uptake was inhibited to a greater extent than was the acetate utilization (Table IX). This indicated a possible accumulation of intermediates between acetate and the locus of action of the inhibitor. In the malonate-inhibited acetate oxidation system, succinate was shown to accumulate and accounted for approximately 55 per cent of the acetate utilized (Table X). This is additional evidence demonstrating the oxidation of acetate via the tricarboxylic acid cycle.

SUMMARY

1. Acetate is completely oxidized by homogenates of rabbit kidney cortex.
2. Mg^{++} or Mn^{++} , inorganic phosphate, adenosinetriphosphate, oxalacetate, and pantothenate are necessary for acetate oxidation by washed preparations.
3. Coenzyme A stimulates the formation of citrate from acetate plus oxalacetate by extracts of acetone powder preparations.
4. The acetate oxidation system is approximately equally divided between the residue and mitochondrial fractions.
5. Malonate, arsenite, iodoacetate, and fluoroacetate inhibit acetate oxidation. Succinate accumulates from acetate in the malonate-inhibited acetate oxidation system.

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¹ Novelli and Lipmann's excellent and comprehensive paper (35) on the catalytic function of coenzyme A in citric acid synthesis appeared during the preparation of this manuscript.

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A MECHANISM FOR FLUOROACETATE INHIBITION*

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The toxic nature of fluoroacetate and its derivatives was first reported by McCombie and Saunders (1, 2). They pointed out that substances capable of yielding fluoroacetate on oxidation in the animal were also toxic. Other workers (3) investigated the effects of fluoroacetate on a number of enzyme systems, and, on the basis of its action on acetate oxidation, postulated that the toxicity of fluoroacetate was due to competitive inhibition of acetate oxidation. Additional evidence in support of this theory was presented in studies on the effects of fluoroacetate on the metabolism of acetate and pyruvate in yeast and bacteria (4) and the effect of fluoroacetate and fluorobutyrate on fatty acid and glucose oxidation by kidney homogenates (5).

However, the above hypothesis does not satisfactorily explain some of the enzymatic effects obtained with this inhibitor. With fluoroacetate-poisoned kidney preparations, there is a marked accumulation of citrate in the presence of oxalacetate (5-7) or of fumarate (8) without an accompanying accumulation of acetate. Citrate also accumulates in tissues of animals poisoned with fluoroacetate (9). At low concentrations of fluoroacetate which markedly inhibit acetate oxidation by kidney preparations, citrate oxidation is much less affected (6, 8). However, citrate utilization by preparations of heart muscle is inhibited by fluoroacetate, whereas the condensation of "active" acetate with oxalacetate is not inhibited (10). With the filarial worm, *Lilomosoides carinii*, there is no evidence of a competitive inhibition of acetate oxidation, although respiration is inhibited (11). *In vivo*, there is a latent period between the administration of the drug and the development of the characteristic toxic symptoms (12). It has been suggested by Liebecq and Peters (8) and by Martius (10) that fluoroacetate is not the toxic compound but that it is transformed to another substance, possibly fluorocitrate, which is inhibitory.

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The present communication presents evidence for the formation of a fluorocitrate, small amounts of which competitively inhibit citrate oxidation by a preparation of washed rabbit kidney cortex.

Methods

Tissue fractionation and manometric determinations were carried out as described in a previous article (13). Citrate was determined as previously described (6). Citrate was isolated as the calcium salt by precipitating it in the presence of excess citrate with boiling 50 per cent calcium chloride and washed twice with large volumes of boiling distilled water. Fluoride was determined by the method of Benedetti-Pichler (14), after

TABLE I
Inhibition of Acetate Oxidation by Fluoroacetate and Reversal by Oxalacetate

Experiment No.	Fluoroacetate	Oxalacetate, added	Oxygen uptake
	<i>M</i>	<i>μM</i>	<i>μl.</i>
1	0.001		42
	0.001	5	276
	0.001	20	220
2			220
	0.003		45
	0.003	5	85
3	0.01	10	19
	0.01	50	73

Flask contents, residue washed four times 0.4 ml., MgCl_2 0.01 M, Na_2HPO_4 0.01 M, adenosinetriphosphate 0.001 M, pantothenate 150 γ , acetate 100 μM . Fluoroacetate was incubated with the tissue preparations for 15 minutes before acetate was tipped in from the side arm. Total volume, 3.0 ml. plus KOH in center well. Time, Experiments 1 and 3, 70 minutes; Experiment 2, 120 minutes.

sodium fusion (15) of the compounds being tested. *cis*-Aconitic anhydride was prepared from *trans*-aconitic acid (16). The anhydride was dissolved and neutralized immediately before use. Oxalacetic acid was prepared by the method of Krampitz and Werkman (17). Sodium monofluoroacetate was obtained through the courtesy of Dr. J. O. Hutchens, Director of the Toxicity Laboratory of the University of Chicago.

Results

Effect of Oxalacetate on Fluoroacetate Inhibition of Acetate Oxidation

Fluoroacetate largely inhibited acetate oxidation as expected, but added oxalacetate reversed the inhibition, especially at lower concentrations of the inhibitor (Table I). This would not be expected if fluoroacetate were acting competitively with acetate. The fluoroacetate inhibition was

slowly released with time, thus corresponding to an induction period similar to the one described by Lynen (18) for the oxidation of acetate by starved yeast. The addition of $5\text{ }\mu\text{M}$ of oxalacetate sharply decreased the inhibition or duration of the induction period due to fluoroacetate (Fig. 1). Therefore, the effect of fluoroacetate seems to be largely due to a deficiency of oxalacetate in the poisoned cell and not to a competition with acetate. The major difference between these experiments and the experiments on which the theory of competitive inhibition of acetate was based was in the addition of oxalacetate.

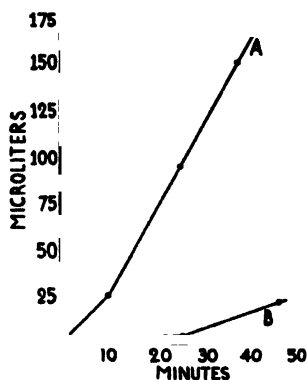


FIG. 1

FIG. 1. The effect of fluoroacetate on the induction period. Curve A, acetate $100\text{ }\mu\text{M}$, fluoroacetate 0.001 M , and oxalacetate $5\text{ }\mu\text{M}$; Curve B, acetate $100\text{ }\mu\text{M}$, and fluoroacetate 0.001 M . Other flask contents as in Table I.

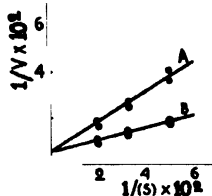


FIG. 2

FIG. 2. Competitive inhibition of citrate oxidation by fluoroacetate. Curve A, citrate plus 0.003 M fluoroacetate; Curve B, citrate. Other flask contents as in Table II. Time, 75 minutes.

Effect of Fluoroacetate Plus Oxalacetate on Oxidation of Citrate

The probable effect of oxalacetate in this system would be to catalyze the initial step in the utilization of acetate via the Krebs cycle; namely, the formation of citrate. It seemed possible that, as suggested by Martius and as indicated by the results of Liebecq and Peters, fluoroacetate might also condense with oxalacetate to form a fluorocitrate which would inhibit citrate oxidation and account for the accumulation of citrate in fluoroacetate-poisoned systems. Accordingly, $3\text{ }\mu\text{M}$ of fluoroacetate (0.001 M) and $5\text{ }\mu\text{M}$ of oxalacetate were incubated with the tissue for 15 minutes, after which *small amounts* of citrate were added. The resulting oxidation of the citrate under these conditions was inhibited 83 per cent (Table II). With larger amounts of citrate the inhibition was appreciably reversed.

When the reciprocal of the reaction velocity ($1/v$) and the reciprocal of the substrate concentration ($1/S$) were plotted for a number of concentrations of citrate (20, 30, and 50 μM) with and without 0.003 M fluoroacetate, the graph in Fig. 2 resulted. This type of graph is indicative of competitive inhibition (19).

TABLE II
Effect of Fluoroacetate on Citrate Oxidation

Citrate	Fluoroacetate, 3 μM	Oxygen uptake	Inhibition
μM		$\mu\text{l.}$	<i>per cent</i>
10	—	84	
10	+	14	83
20	+	14	83
30	+	46	45
50	+	63	25

Flask contents, residue washed four times 1.0 ml., MgCl_2 0.01 M, Na_2HPO_4 0.01 M, adenosinetriphosphate 0.001 M, oxalacetate 5 μM , citrate and fluoroacetate as indicated, Sørensen's glycine buffer (pH 8.2) 0.4 ml. Total volume, 3 ml. plus KOH in center well. Time, 45 minutes. Fluoroacetate and oxalacetate were incubated with the tissue for 15 minutes before the citrate was tipped in from the side arm.

TABLE III
Effect of Fluoroacetate on Citrate and cis-Aconitate Oxidation

Substrate, 10 μM	Fluoroacetate, 9 μM	Oxygen uptake	Inhibition
		$\mu\text{l.}$	<i>per cent</i>
<i>cis</i> -Aconitate.....	—	86	
“.....	+	108	0
Citrate.....	—	140	
“.....	+	9	94

Conditions as in Table II.

Effect of Fluoroacetate on cis-Aconitate Oxidation

A number of similar experiments with *cis*-aconitate and isocitrate as substrates showed that the oxidation of *cis*-aconitate and isocitrate was not inhibited by fluoroacetate plus oxalacetate under conditions favoring the formation of a fluoro derivative of citrate. The inhibition of citrate oxidation with 0.003 M fluoroacetate was 94 per cent complete, and in the same experiment *cis*-aconitate oxidation was not inhibited (Table III). Therefore it appears that the inhibition took place at the step between citrate and *cis*-aconitate.

*Isolation of Fluoride from Citrate Formed from Fluoroacetate
and Oxalacetate*

In order to verify the formation of a fluoro derivative of citrate, an experiment in duplicate was set up with 200 μ M each of oxalacetate and fluoroacetate and 5 cc. of residue (washed four times) per flask. 32 μ M of citrate were formed per flask. The protein-free filtrate from one flask was treated with H_2SO_4 , $KMnO_4$, and KBr , the reagents used in the highly specific and quantitative reaction for the conversion of citrate to pentabromoacetone (20, 21). The petroleum ether solution of the pentabromoacetone was washed twice with water and was evaporated by use of a gentle stream of carbon dioxide gas until almost all the petroleum ether was removed. A small amount of alcohol was then added. The alcohol solution of the pentabromoacetone was fused with sodium and the fusion products gave a positive test for fluoride. Control samples to which fluoroacetate and citrate in equivalent quantities were added *after deproteinization* gave no fluoride test after identical treatment. To a second duplicate flask in which citrate had been formed in the presence of fluoroacetate and oxalacetate, an excess of citrate was added as carrier and the citrate isolated as the calcium salt. This citrate was fused with sodium, and the fusion mixture gave a positive test for fluoride. A control sample containing deproteinized tissue, fluoroacetate, and citrate in equivalent quantities gave a negative test for fluoride.

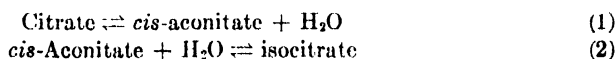
DISCUSSION

There do not seem to be any adequate methods available for the quantitative microdetermination of compounds such as fluoroacetic and fluorocitric acids. To date, nearly all methods depend upon drastic non-quantitative treatment to split the very stable C—F bond and detect the liberated fluoride ion (12). However, the qualitative demonstration of fluoride (limit of identification, approximately 2 γ of F) in the "citrate" formed in the presence of oxalacetate and fluoroacetate, and in the pentabromoacetone derived from this "citrate," indicates the formation of a monofluorocitrate or a closely related compound. It is interesting that as little as 3 to 9 μ M of this compound, representing 57 to 171 γ of F, (assuming maximum conversion from the fluoroacetate present, Tables II and III) could produce such large inhibitions of citrate oxidation.

The observed action of oxalacetate in reversing the inhibition of acetate oxidation caused by fluoroacetate would indicate that the inhibition is due to a shortage of oxalacetate caused by the stoppage of the tricarboxylic acid cycle at the citrate \rightarrow *cis*-aconitate stage. The addition of sufficient oxalacetate to such an inhibited system presumably allows the

formation of sufficient citrate to reverse the inhibition to some degree. It therefore appears that the inhibition of acetate oxidation could be the result of inhibition of citrate oxidation. This would account for the seemingly great sensitivity of pyruvate oxidation to fluoroacetate (22, 23) shown by bakers' yeast and by *Chilomonas paramecium*, for the lack of competitive inhibition of acetate oxidation in filaria (11), for the accumulation of citrate in fluoroacetate-poisoned systems (5-9), and for the latent period always observed *in vivo* (12).

The inhibition of citrate oxidation without concurrent inhibition of *cis*-aconitate oxidation would seem to offer additional evidence that citrate is a precursor of aconitate in the tricarboxylic acid cycle (24) and not vice versa, and that aconitase is *two* enzymes, catalyzing two separate reactions



only the enzyme catalyzing the first reaction being inhibited by fluorocitrate.

Some indirect evidence has previously been presented (25) indicating that aconitase may be two enzymes, β and α , catalyzing Reactions 1 and 2 respectively. However, fractionation and purification (23-fold) of aconitase did not support this conclusion (26). Another interpretation is also possible. *cis*-Aconitate may have a higher affinity for the enzyme aconitase than citrate does. If this were so, the competitive inhibition by fluorocitrate might not be as effective against *cis*-aconitate as against citrate, because the *cis*-aconitate might displace the fluorocitrate from the enzyme, while citrate could not.

SUMMARY

Sodium monofluoroacetate (0.001 M) inhibited acetate oxidation by a preparation of washed rabbit kidney cortex. This inhibition was largely reversed by the addition of 5 μ M of oxalacetate.

Citrate oxidation was inhibited to a similar or slightly greater extent when the tissue was incubated with fluoroacetate *and* oxalacetate for 15 minutes before the addition of the citrate. This inhibition was competitive. Under the same conditions, the oxidation of *cis*-aconitate and isocitrate was not inhibited.

The formation of a fluorocitrate was demonstrated by incubating tissue, oxalacetate, and fluoroacetate, adding normal citrate as a carrier after deproteinization, and demonstrating the presence of fluoride ion in the isolated citrate and in the pentabromoacetone fraction obtained from the citrate.

An explanation of the inhibition of acetate oxidation by fluoroacetate is presented.

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STUDIES ON HYALURONIC ACID AND RELATED SUBSTANCES

I. PREPARATION OF HYALURONIC ACID AND DERIVATIVES FROM HUMAN UMBILICAL CORD

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In studying the chemical structure of hyaluronic acid it has been found necessary to modify existing methods of isolation and determine the degree of purity of the compounds obtained by analysis, electrometric titration, and viscosity determinations.

Two procedures based on the work of Meyer and Palmer (1), Robertson, Ropes, and Bauer (2), and Hadidian and Pirie (3) have been studied. In Method A, the human umbilical cords are digested with pepsin and trypsin, and the crude hyaluronate is precipitated with ethanol and then further purified from the remaining proteins by Sevag's procedure. In Method B, the cords are extracted with a saline solution and the hyaluronate is precipitated with ammonium sulfate and pyridine and then further purified by Sevag's procedure.

By Method B small quantities of a highly pure product, free from traces of enzyme, are obtained with ease and rapidity. With Method A all the available hyaluronic acid is extracted, and it is more suitable for handling large quantities of starting material, but yields a hyaluronate contaminated with a polysaccharide sulfate. This polysaccharide may be removed with pyridine and ammonium sulfate precipitation.

Both viscous-free acid and the sodium salt were prepared and a study was made of various derivatives; the products were obtained by treatment with diazomethane, with acetic anhydride and pyridine, and with triphenylchloromethane and pyridine.

The viscosity of these various derivatives was determined at various concentrations, and the action of heat on pure, highly viscous hyaluronate was studied.

EXPERIMENTAL

Method A. Preparation by Enzymatic Digestion

1. *Digestion of Proteins*—3000 gm. of human umbilical cord, corresponding to 410 gm. of dried, crude material, free from blood and placental

* This investigation was aided by a grant from G. D. Searle and Company.

tissue and stored under acetone for 2 to 10 weeks, were cut into 2 cm. segments and washed with 12 liters of fresh acetone. The washing was repeated twice with 12 liters of distilled water, each with a 2 to 4 hours soaking period, and completed with a third portion of distilled water and soaking for 18 to 24 hours. The cords were passed through a power-driven meat grinder and diluted with an equal volume of distilled water; the pH was adjusted to 2.0 by the addition of 300 ml. of 2 N HCl. 9 gm. of pepsin (Difco, 1:10,000) were added and the mixture, protected by a layer of toluene, was incubated for 24 hours at 37°. The pH was checked and adjusted at frequent intervals during the incubation. After 24 hours, the pH was raised to 7.4 with 90 ml. of 10 N NaOH, 16 gm. of trypsin (Difco, 1:250) were added, and the mixture was again incubated at 37° for 24 hours. After the digestion was completed, the toluene was separated and the residual mixture centrifuged for 30 minutes.¹ The precipitate was discarded, the solution cooled at 5° was acidified to pH 2.0 with 5 N HCl, 2 volumes of 95 per cent ethanol were added to the supernatant liquid,² and the mixture was centrifuged. The precipitate was suspended in about 1000 ml. of distilled water, placed in cellophane membranes, and dialyzed against running tap water for 24 hours.

2. *Elimination of Residual Proteins by Sevag's Procedure*—A mixture of 3.3 liters of chloroform and 1.7 liters of amyl alcohol and 1 liter of a water solution containing 300 gm. of sodium acetate and 160 gm. of glacial acetic acid was added to the dialyzed solution (volume, 5 liters). This mixture was shaken vigorously in a mechanical shaker for 10 minutes and then centrifuged for 30 minutes. The supernatant aqueous phase

¹ In a similar experiment, starting with 1200 gm. of human cord, centrifugation was substituted by filtration on a Büchner funnel, with Whatman No. 4 filter paper prepared with a filter aid (Celite). The hyaluronic acid solution obtained was subjected only two or three times to the Sevag procedure and dialyzed as in previous experiments. It was then precipitated with 2 volumes of 95 per cent ethanol, washed twice with 95 per cent alcohol, and thrice with ether and dried at room temperature. The last traces of ether were removed by drying in a vacuum desiccator (Fraction II). The yield was 12.5 gm.

In another similar experiment, starting with 700 gm. of cord, the centrifugation was carried out at 20,000 r.p.m. in a Sharples centrifuge. The hyaluronate (Fraction III₂) was precipitated with alcohol, washed with alcohol, and ether-dried as above to yield 7.0 gm. of hyaluronate. The analysis of Fractions II and III and the viscosity determinations before precipitation indicate that a portion of the hyaluronic acid is lost during the isolation. In one instance the most viscous fractions remained on the filter, and, in the other instance, the most viscous element was retained in the rotor of the Sharples centrifuge along with the proteins. Therefore, the proportion of polysaccharide sulfate is increased through loss of hyaluronic acid, as is also shown by the higher sulfur content and lower viscosity.

² At this point the mixture was preferably deaerated by evacuation, since this facilitated the settling of the precipitate.

containing the hyaluronic acid was siphoned and treated repeatedly with the same mixture of chloroform and amyl alcohol until no more precipitate appeared at the interface. 2 volumes of 95 per cent alcohol were added to the aqueous phase containing the hyaluronic acid (volume, 6 liters). The precipitate was centrifuged for 10 to 15 minutes, dissolved in distilled water, dialyzed against running tap water for 24 hours, and then against several changes of distilled water for 24 hours. The 4 liters of solution containing 29 gm. of hyaluronate (Fraction I₈)³ were saturated with chloroform after dialysis and stored at 5°. It contains in general about 20 per cent of polysaccharide sulfate and can be purified by the application of the second method (B4), affording a yield of 5.6 per cent of pure hyaluronate based on the weight of dried cords.

Method B. Preparation by Precipitation with Ammonium Sulfate and Pyridine

1. *Saline Extraction*—Human umbilical cords (1400 gm. or 190 gm. of dry material) were collected, washed, and prepared the same way as in Method A1. The material was ground and suspended in 4 liters of 0.1 M sodium chloride solution saturated with chloroform. This extraction was allowed to proceed in the cold for 24 hours with occasional stirring. The mixture was placed in a cloth bag and the fluid was expressed. After a second extraction of the residue in the same way, the combined extracts were acidified at 5° to pH 1.5 to 2.0 with 180 ml. of 5 N HCl and centrifuged. The precipitates were added to the residue and set aside for enzymatic digestion (Method B4).

2. *Precipitation with Ammonium Sulfate*—To the solution were added, with vigorous stirring, 2700 gm. of ammonium sulfate and the mixture was kept for precipitation at about 5°, which was complete after several hours. After centrifugation, the solution was treated according to Method B3 and the precipitates were dissolved in water and dialyzed against running tap water for 24 hours and then against distilled water for 24 hours. This solution was treated according to the Sevag technique (Method A2) and afforded 3 gm. of pure hyaluronate (Fraction IV₈) in solution.

3. *Precipitation with Pyridine*—To the ammonium sulfate solution from above were added 500 ml. of pyridine, with vigorous stirring, and the mixture was allowed to stand for about 4 hours. To complete the precipitation 2500 gm. of ammonium sulfate were added slowly, with vigorous stirring, and the mixture was refrigerated for several hours. The

³ Fractions are identified by roman numerals and their form or derivatives by a letter (e.g., Fraction I in solution I₈, lyophilized I_L; the free acid prepared from Fraction I, I_A, etc.).

bulk of the precipitate rose to the interface, some precipitate adhering to the sides of the vessel. The subjacent liquid was siphoned, as completely as possible, and the precipitate, along with the pyridine, was centrifuged for 30 minutes to 1 hour. The pyridine was siphoned and the pellicles formed at the interface were washed with 95 per cent alcohol and centrifuged. The pellicles were cut into small pieces, suspended in distilled water, and dialyzed against tap water for 24 hours and then against distilled water for 24 hours. The material was further purified by the Sevag procedure as described in Method A2. After final dialysis, the solution (500 ml.) contained 1 gm. of hyaluronate (Fraction V₈).

An additional 1200 gm. of ammonium sulfate were added to the ammonium sulfate-pyridine solution and the mixture was treated as described in the preceding fractionation. This makes certain that no hyaluronic acid remains in the ammonium sulfate liquor. If a precipitate was present, the material was treated as in the previous extraction.

4. *Extraction and Purification of Residual Hyaluronic Acid*—The combined cord residues were purified by enzymatic digestion as described in Method A1. The final solution (975 ml., Fraction VI₈) obtained contained 7 gm. of impure hyaluronate, contaminated with about 30 per cent of polysaccharide sulfate and 5 to 10 per cent of proteins. This solution of hyaluronate was purified by a new precipitation with ammonium sulfate in the presence of pyridine as follows.

500 gm. of ammonium sulfate and 50 ml. of pyridine were added with vigorous agitation. The mixture was allowed to stand for 1 to 2 hours in the cold and then centrifuged for 1 hour. The pellicle formed was washed with alcohol, centrifuged, cut up, suspended in water, and dialyzed for 24 hours against running tap water. This hyaluronic acid solution was treated a second time with ammonium sulfate and pyridine to insure complete removal of the sulfur-containing compound, then completely dialyzed; it contained 4 gm. of hyaluronate (Fraction VII₈). The combined yield of all the fractions was 8 gm. or 4.2 per cent, based on the weight of the dried cords.

The two ammonium sulfate-pyridine liquors were dialyzed in a similar manner and the dialyzed solution was concentrated by lyophilization, yielding 2.5 gm. of residue.

Analysis—Nitrogen (Kjeldahl) 2.90, acetyl 7.4, sulfur 3.8

The study of this sulfur-containing polysaccharide which was found as the contaminant of hyaluronic acid isolated from human umbilical cord will be described in a forthcoming publication.

Analysis—The analyses were performed either on hyaluronic acid in solution (exact concentration established by drying an aliquot to constant

weight), on lyophilized, or alcohol-precipitated and ether-dried samples. The analyses obtained from these three types of samples were all identical.

The absence of glycogen in each fraction was demonstrated by the absence of a color reaction with iodine.

Water Content—It is very difficult to remove moisture completely from dried hyaluronic acid, as was previously observed by Meyer and Palmer (1) and as is the case with numerous other polysaccharides. Our samples have been dried to constant weight *in vacuo* at 115° in the presence of phosphoric anhydride for a minimum period of 15 hours. The samples were weighed in closed vessels.

Acetyl—The determinations were made after acid digestion, according to Hadidian and Pirie (3), with the apparatus described by Markham (4).

TABLE I
Analysis of Various Hyaluronate Fractions

Preparation No.	Nitrogen		Ash as		Acetyl	Sulfur
	Kjeldahl	Dumas	SO ₄ ²⁻	Na		
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Sodium hyaluronate (theoretical)	3.49	3.49	19.70	5.73	10.73	0.0
I	3.40	3.04	18.2		9.6	1.2
II	3.74	2.63	21.0	6.5		1.5
III	4.27	2.85	18.2	5.3	9.5	2.0
IV	3.64	2.36	14.6	4.15	10.2	0.10
V	3.45	2.28	15.4	4.65	10.4	0.14
VI	4.15	2.94	17.5	2.4	9.6	1.67
VII	3.64	2.94			10.0	0.16

Nitrogen—Determinations were made according to the Kjeldahl and Dumas methods. The former method produced consistent results and the latter, as is the case with many other natural products, gave results that were too low and inconsistent.

Sulfur—The determination was carried out by the combustion procedure of Pregl, as described by Niederl and Niederl (5). The residue from the combustion, due to the presence of sodium, was included in the sample to be precipitated.

Metals—The metals present were converted to the sulfate by incineration after the addition of sulfuric acid. The salts were then dissolved in a large volume of water and sodium was determined by a flame photometer (Table I).

⁴ Nitrogen (Dumas method), sulfur, and methoxyl were determined by the Analytical Division of G. D. Searle and Company, and by F. Weiser, Basel, Switzerland.

Derivatives

Free Acid and Sodium Salt—To test the hypothesis that anhydride linkages exist between the chains of high viscosity hyaluronic acid (6), the free acid was prepared from the hyaluronate and its neutral equivalent determined by electrometric titration according to Unruh *et al.* (7). At the same time the viscosity was compared with that of the starting material. The free acid has previously been prepared by precipitation with glacial acetic acid (1). The partial degradation which always accompanied this step vitiated any viscosity comparison with the starting material. We have obviated this difficulty in the preparation of the free

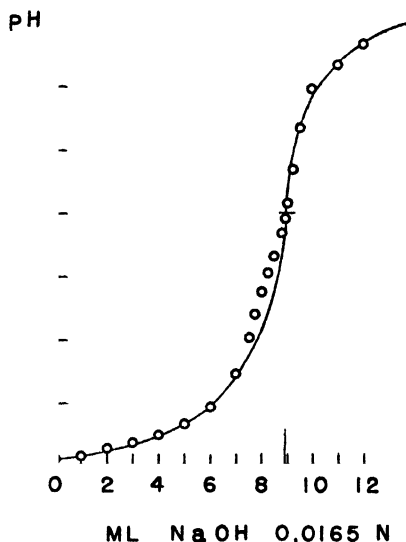


FIG. 1. Electrometric titration of 58.0 mg. of Hyaluronic Acid IV_A with 0.0165 N sodium hydroxide in 1 N sodium bromide solution.

acid by passing a solution of hyaluronate through a column of synthetic ion exchange resin, a method which has been applied to other water-soluble polyuronides (8).

Procedure—Through a column of Dowex 50⁵ 20 cm. high and 3 cm. in diameter, previously washed with 200 ml. of 2 N sulfuric acid, and then 2000 ml. of distilled water, 120 ml. (containing 0.40 gm.) of hyaluronate solution (Fraction IV_B) were passed under a slight pressure of nitrogen. The column was washed with 60 ml. of distilled water; further washing was not done in order to avoid dilution. The percolate (200 ml.) contained 0.290 gm. of free Hyaluronic Acid IV_A. To 40.0 ml. of this solu-

⁵ This product was kindly furnished by The Dow Chemical Company, Midland, Michigan.

tion were added 20 ml. of 2 N sodium bromide solution plus 40 ml. of distilled water and the free acid was titrated with 0.0165 N sodium hydroxide solution with glass and calomel electrodes in the Beckman model G pH meter. The results are shown in Fig. 1.

After the titration, the solution containing the sodium salt and a slight excess of NaOH was dialyzed in the cold for 2 days against repeated changes of distilled water and then lyophilized to residual Sodium Hyaluronate IV_N. Hyaluronic Acid I_A was prepared in the same manner starting from Hyaluronate I_S.

Analysis—

Hyaluronic Acid.	Calculated.	Ash 0,	neutral equivalent	379.3
" " I _A .	Found.	" 1.6,	" "	382
" " IV _A .	"	" 1.3,	" "	395
Sodium Hyaluronate IV _N .	Calculated.	N 3.49, acetyl 10.73, ash 17.70, Na 5.79		
	Found.	" 3.52,* " 9.10, " 16.6, " 5.1		

* Kjeldahl.

Methyl Ester of Hyaluronic Acid—50 ml. of solution containing 75 mg. of free Hyaluronic Acid IV_A (pH 3.4) were shaken in a separatory funnel with three 10 ml. portions of 2 per cent diazomethane in ether until there was no further evolution of gas. The ether layer was discarded and the aqueous solution, now at pH 6.8, dialyzed against distilled water for 24 hours in the cold (5°). Part of the solution of Methyl Hyaluronate was lyophilized for analysis and the remainder used for a viscosity determination.

Analysis—Methoxyl. Calculated, 7.9; found, 5.35, 5.29⁶

Acetyl Hyaluronate—In order to estimate the molecular weight by osmotic pressure measurements in chloroform or benzyl alcohol solution and gain some idea of the shape of the molecules by viscosity determinations, an acetyl derivative was prepared. To avoid any degradation the acetylation was carried out in a medium of anhydrous pyridine and acetic anhydride, after the hyaluronic acid had been swollen in formamide, according to the method described by Hadidian and Pirie (9).

2 gm. of lyophilized Hyaluronate I_L were dried for 2 hours at 120° *in vacuo* in the presence of phosphoric anhydride, then dispersed in 80 ml. of formamide.⁷ 80 ml. of anhydrous pyridine were added and, after

⁶ The analysis refers to the repeating period, *i.e.*, 1 molecule of *N*-acetylglucosamine and 1 molecule of glucuronic acid less 2 molecules of H₂O.

⁷ Contrary to the observations of Hadidian and Pirie (9), the dispersion in formamide is not dependent on the temperature at which it occurs but rather on the physical state of hyaluronic acid. A product lyophilized in a very dilute solution and with a very porous structure will become dispersed very readily within a few minutes at room temperature, no particles remaining visible, whereas products

being thoroughly mixed with 80 ml. of acetic anhydride, the solution was allowed to stand at 25° for 40 hours. The resulting deep brown solution was then poured on 300 gm. of ice and immediately dialyzed, first against running tap water until the acetic acid and pyridine were completely removed, then against distilled water for 24 hours. After centrifugation for 30 minutes, 0.17 gm. of insoluble Acetate A was recovered. The solution was lyophilized to yield 1.5 gm. of pale yellow soluble Acetate A.

The acetylation was also carried out for 1 hour at 80°. The products obtained, Acetates B, are dark green in color. After lyophilization, the solubility of these acetates is greatly diminished.

<i>Analysis</i> —Di- <i>O</i> -acetyl hyaluronate.		Calculated.	Acetyl	26.63,	Ash	14.63
Tri- <i>O</i> -acetyl	"	"	"	32.66,	"	13.47
Acetate A, soluble.		Found.	"	29.6,	"	11.4
" " insoluble.		"	"	30.0,	"	10.9
" B, soluble.		"	"	26.4,	"	15.6
" " insoluble.		"	"	29.9,	"	11.2

The products correspond to mixtures of di- and triacetates. Soluble Acetate A (0.27 gm.), finely suspended in 1000 ml. of distilled water, was passed through a column of Dowex 50. The suspension was lyophilized and the residue, which contained only 0.5 per cent ash, was resuspended in 100 ml. of water. This suspension was then agitated in a separatory funnel with 50 ml. of a 2 per cent ether solution of diazomethane, added in three portions. The acetylhyaluronic acid dissolved completely in the water. The ether layer was discarded and the last traces of ether were removed from the aqueous layer by evacuation. The solution was lyophilized and yielded 0.270 gm. of residual product.

Analysis—Methoxyl. Calculated, 5.95; found, 0.65, 0.57

This product was dispersed in 20 ml. of pyridine, and 10 ml. of acetic anhydride were added. After 2 days of contact at room temperature, the suspension was heated for 2 hours at 100° with moisture protection, cooled, and poured on to 100 gm. of ice. A resulting slight precipitate which formed was centrifuged; the centrifugate was dialyzed against running tap water, then against distilled water until all the pyridine and acetic acid were completely removed, and the dialyzed solution was finally lyophilized.

Analysis—Acetyl. Found, 23.6

It was not possible, therefore, to increase the proportion of acetyl groups by acetylation in the presence of pyridine. The same experiment

dried in the form of films remain unchanged in contact with formamide, even after several days at a slightly elevated temperature (about 50°).

was performed on the soluble Acetate B with the same result. The products were insoluble in organic solvents, such as benzyl alcohol, chloroparaffins, acetone, and pyridine, and thus it was not possible to make physicochemical measurements.

Trityl Hyaluronate—This compound was prepared by the method of Low and White (10), in an attempt to determine whether position 6 of the glucosamine moiety was free or glucosidically linked.

500 mg. of lyophilized Hyaluronate I_L, dried in the same manner as the material which was used for acetylation, were introduced in a tube with 10 ml. of anhydrous pyridine and 2 gm. of triphenylchloromethane. The tube was sealed and shaken for 20 hours at 55°, followed by 2 hours at 100°. Only a small part dispersed and seemingly entered into the reaction. The contents of the tube were cooled and then poured into 20 ml. of anhydrous acetone. This mixture was filtered and the precipitate washed thoroughly with acetone and dried by exposure to air; it was then left in contact with distilled water in the cold for 1 week. The insoluble residue was filtered, carefully washed with water, and the washings were collected and lyophilized. The residual gray powder, which weighed 0.130 gm. after desiccation over calcium chloride, contained 0.53 trityl groups per repeating unit period.⁸

The lyophilized aqueous solution yielded 0.390 gm. of hyaluronic acid in which no trityl groups could be found.

0.5 gm. of dried, lyophilized Hyaluronate I_L was dispersed in 30 ml. of formamide, to which 25 ml. of pyridine and 2 gm. of triphenylchloromethane were added. The mixture was kept at 50° for 24 hours, protected from moisture, and, after cooling, poured into 200 ml. of acetone and treated as above. 50 mg. of insoluble product, containing 0.95 trityl group per repeating unit, were isolated. No trityl groups could be detected in the hyaluronic acid obtained from the aqueous solution.

Viscosity—The viscosity was determined with an Ostwald viscosimeter having a capillary approximately 9 cm. long and a flow time of 30 to 35 seconds for distilled water. A total volume of 4.0 ml. was used for each determination which was run at 25° in 0.05 M sodium chloride solution buffered at pH 7.0 with phosphate (11, 3).

In order to obtain data on the shape of the molecule and on the factors which affect viscosity, the measurements were made with varying concentrations, and curves of the intrinsic viscosity (ratio of specific viscosity to concentration) as a function of concentration were established.

* The trityl content was determined by hydrolysis in hot, concentrated sulfuric acid followed by dilution with water, filtration, washing with water, and drying; the insoluble triphenylcarbinol, dried to constant weight, was identified by its melting point.

Viscosity of Various Fractions—The various fractions obtained (Fractions I, IV, V, VII) by the procedures described showed practically the same high values for viscosity when determinations were made before lyophilization or precipitation and drying (Fig. 2). When the viscosity is low, this is due to the presence of more or less large quantities of polysaccharide sulfates, as shown by sulfur analysis, as the viscosity of chondroitinsulfuric acid solution is very low (12) in comparison with that of hyaluronic acid. The viscosities of hyaluronic acid isolated by the various methods are all much greater than those reported by Blix and Snell-

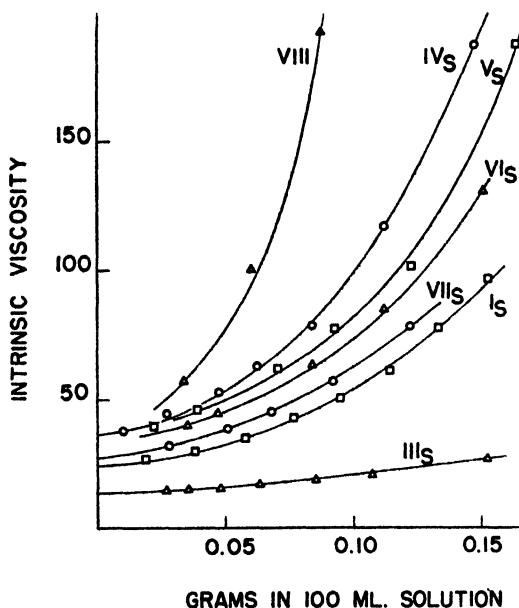


FIG. 2. Intrinsic viscosities of solutions of hyaluronate fractions before lyophilization or precipitation (Curves I_s and III_s to VII_s). Intrinsic viscosity of pleural fluid calculated from the data of Meyer and Chaffee (13) (Curve VIII).

man (12) for their product, which indicates a molecular weight or weight of the micelle of aggregation many times greater than that found by these authors. The relatively low viscosity of their products may be due to incomplete extraction, as in the case of Fraction III described above.

An attempt to purify Fraction IV further through fractionation by centrifugation at 20,000 r.p.m. for 3 hours gave no conclusive results.

The various fractions represent the total hyaluronic acid contained in umbilical cords. Their relative viscosity is very high compared to the other hyaluronic acid described.⁹ (The relative viscosity of Fraction V at

⁹ See summary in Hadidian and Pirie (3) and Meyer (6).

a concentration of 0.3 gm. per 100 ml. = 485; and at 0.1 gm. per 100 ml. = 10.) Pleural fluid has an intrinsic viscosity, calculated with the data of Meyer and Chaffee (13) for an infinite dilution, approximately the same as that of the hyaluronic acid described in this paper, but the increase as a function of concentration is much greater (Curve VIII, Fig. 2). This indicates that if the viscosity of the fluid is indeed due to hyaluronic acid the aggregation of the latter is much greater in its native state and is partially destroyed in the course of isolation.

Ogston and Stanier (14) recently obtained from synovial fluid a complex of hyaluronic acid and protein which possessed a relative viscosity of 39 at the concentration of 0.1 gm. per 100 ml. and was homogeneous in the ultracentrifuge. It is therefore probable that the proteins are responsible for the high viscosity of fluid containing hyaluronic acid in its native state and that any purification to obtain the polysaccharide protein-free should cause degradation of the aggregate of particles.

Influence of Time and Temperature—Numerous observations on the stability of hyaluronic acid in the presence of oxygen and oxidation-reduction systems have been published (15). In this paper, only the influence of time and temperature on viscosity has been studied (Fig. 3).

Precipitation with 95 per cent ethanol of Fraction V_8 followed by ether washing and finally drying at room temperature does not affect the viscosity (Curve V_1 , Fig. 3). The viscosity is reduced by lyophilization and more so when the solution is in a more dilute state (Curves V_2 and V_3).

When hyaluronate is lyophilized or obtained by alcohol precipitation and dried to constant weight in a vacuum at 100°, a readily soluble product is obtained. Its viscosity is greatly reduced (Curve V_4 , Fig. 3), especially at high concentration, indicating a "denaturation" of the micelle in which the long filaments become shorter and more compact. The viscosity at infinite dilution is always relatively high, which is possibly due to the interparticle interaction. The "denaturation" is not reversible, for a product left in solution in the presence of salt for 15 days at about 5° did not regain its viscosity (Curve V_5).

Since dried products heated in the presence of air or oxygen-free nitrogen were equally degraded, it appears that oxygen is not the determining factor under these conditions.

A sterile, dialyzed solution retained the same viscosity after being stored for 6 months at 5°, but its viscosity was diminished in the presence of the buffer used for the viscosity measurement (Curve V_6 , Fig. 3).

The degradation depends primarily on the temperature (Curves V_7 and V_8 , Fig. 3). The viscosity decreases slowly as the temperature is raised to 90° and above this threshold value it decreases very rapidly

(Fig. 4). The rate of degradation is initially constant but decreases after 2 hours (Fig. 5).

No evolution of carbon dioxide occurs during this loss in viscosity and acetyl and nitrogen analysis and periodate consumption¹⁰ before and after do not differ.

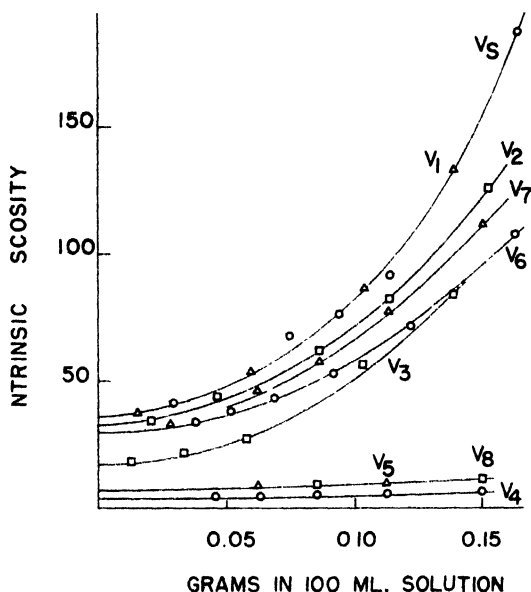


FIG. 3. Influence of precipitation, lyophilization, and heat on the intrinsic viscosity. Hyaluronate V_8 precipitated with ethanol and dried with ether (Curve V_1). Hyaluronate V_8 lyophilized from a solution with a concentration of 0.4 gm. in 100 ml. (Curve V_2). Hyaluronate V_8 lyophilized at a concentration of 0.12 gm. in 100 ml. of solution (Curve V_3). Lyophilized hyaluronate dried at 100° for 24 hours under a high vacuum (Curve V_4). Dried hyaluronate after standing 15 days in buffered 0.05 M sodium chloride at 5° (Curve V_5). Hyaluronate V_8 after standing 30 days in buffered 0.05 M sodium chloride solution at 5° (Curve V_6). Hyaluronate V_8 heated at 60° for 1 hour in the presence of nitrogen (Curve V_7). Hyaluronate V_8 heated at 100° for 1 hour in the presence of nitrogen (Curve V_8).

Influence of Substitution of Reactive Groups—The viscosity of the free Hyaluronic Acid IV_A (Curve IV_1 , Fig. 6) obtained from Fraction IV_8 , in the presence of the previously used buffer, is identical with that of the starting material (Curve IV_8). However, the viscosity of the sodium salt is slightly lower, which is probably due to the slight excess of alkali required to insure complete conversion to the salt and also to the various subsequent treatments (Curve IV_4).

¹⁰ Jeanloz, R. W., and Forchielli, E., unpublished work.

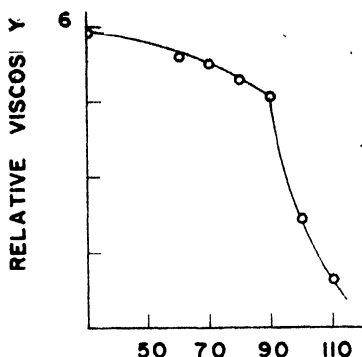


FIG. 4

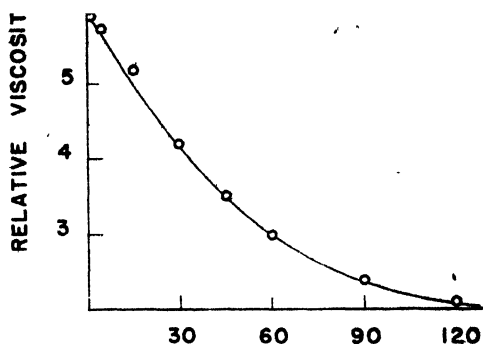
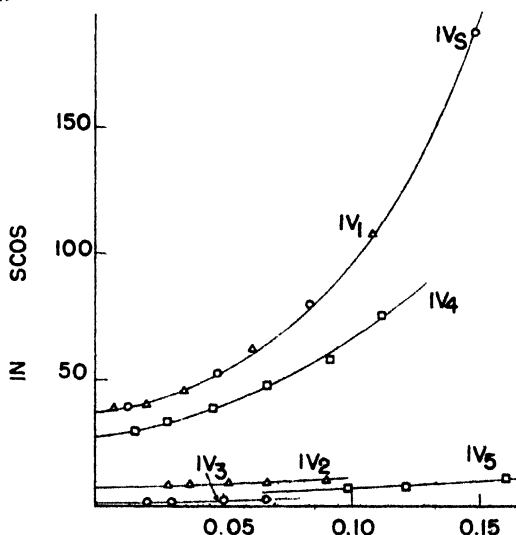


FIG. 5

FIG. 4. Influence of temperature on the relative viscosity. Hyaluronate VII₈ in water heated at various temperatures for 1 hour at a concentration of 0.09 gm. in 100 ml. of solution.

FIG. 5. Influence of time and temperature on the relative viscosity. Hyaluronate VII₈ in water heated at 100° for variable periods at a concentration of 0.09 gm. in 100 ml. solution.



GRAMS IN 100 ML. SOLUTION

FIG. 6. Influence of substitution of the reactive groups on the intrinsic viscosity. Hyaluronic Acid IV_A (Curve IV₁) obtained from Hyaluronate IV₈. Methyl Hyaluronate IV₈ (Curve IV₄). Water solution of 11.3 mg. of Methyl Hyaluronate IV₈ in 10 ml. mixed with 2.5 ml. of 0.016 N sodium hydroxide left standing $\frac{1}{2}$ hour at 25° and then acidified with 1 ml. of 0.1 N acetic acid (Curve IV₂). Sodium Hyaluronate IV_N obtained by neutralization of Hyaluronic Acid IV_A (Curve IV₃). Acetate of Hyaluronate I (Curve IV₅).

Esterification of the carboxyl group causes degradation of the micellar structure (Curve IV₂, Fig. 6); the viscosity is not regained by treating with alkali but, on the contrary, it is considerably lowered (Curve IV₃). The same two-step decrease in viscosity is observed when the hydroxyl groups are acetylated (Curve IV₄) and the acetyl groups subsequently removed under mildly alkaline conditions (η intrinsic 4.0 for a concentration of 0.15 gm. per 100 ml.).

DISCUSSION

The above data show that it is possible to extract hyaluronic acid completely from human umbilical cord and to obtain it in a state of purity of about 95 per cent as indicated by the sulfur analysis and the electro-metric titration. The best method for obtaining large quantities of such a product is a combination of degradation of the proteins by enzymatic digestion (Method A), followed by fractionation with ammonium sulfate in the presence of pyridine (Method B4). The yield, based on dried cord, can be as much as 5 to 6 per cent.

To obtain an enzyme-free product, which is desirable for the assay of hyaluronidase, it is necessary to extract with an aqueous salt solution (Methods B1 and B2) but large quantities of hyaluronic acid would still remain in the cords, being mechanically retained by the proteins.

The elimination of the sulfate-containing polysaccharide is important, for it is present in relatively large proportion (about 20 per cent) and, in the case of the hyaluronidase assay, it has been shown that chondroitin-sulfuric acid and heparin can function as inhibitors (16). This purification step seems to have been generally ignored, for in most of the preparations described in the literature a high sulfate content is indicated (3, 18).¹¹ In contrast to those of Hadidian and Pirie (3), all the fractions obtained by precipitation with ammonium sulfate with or without the presence of pyridine have always contained less than 0.2 per cent of sulfur, corresponding to less than 4 per cent of chondroitin-sulfuric acid. Further, all the fractions after enzymatic digestion had a high acetyl content, the low content of Fraction I being explained by the relatively strong contamination with polysaccharide sulfate. The differences in the salt values depend on the process of purification: in the first method, the pH is at times slightly higher than 7, which causes complete neutralization of the hyaluronic acid, whereas in the second method the working pH is always below 7.¹² Also in contrast to the observation by

¹¹ One single method for purification has been described by Meyer *et al.* (17).

¹² Fractions II, III, and VI contain a low proportion of proteins; these impure products (with large proportions of polysaccharide sulfate) were submitted only two or three times to the Sevag procedure.

Lundquist (19), the viscosities of the fractions obtained through enzymatic digestions were never unstable.

Hyaluronic acid prepared by precipitation in the presence of acid is always contaminated by salt and the neutral equivalents observed for such preparations are always greater than 500 (3, 2, 20). Passage of the sodium salt through a column of acid ion exchange resin readily converts it to the free acid with a minimum of ash content. A sample of hyaluronic acid, so prepared, which had a very high viscosity and yielded a sodium salt of the expected composition, was found to have a neutral equivalent of 395, close to the theoretical value of 379. Therefore, it is necessary to reject Meyer's hypothesis, according to which the high viscosity is due to anhydride linkages between the chains (6). If the small discrepancy of 5 per cent between the actual results and those calculated should indicate the proportion of carboxyl groups chemically combined, it more reasonably may be attributed to ester linkage with hydroxyl groups of another chain.

From the viscosity measurements it follows that the molecular chains of hyaluronic acid are joined in the native state in micelles of a molecular weight of several million. The depolymerization of these large aggregates occurs at the time of extraction regardless of the precautions taken. The most depolymerized being the most dispersible, a rapid and incomplete extraction yields products of relatively low viscosity. If, however, the extraction is pushed to completion, as in the instance of enzymatic digestion of proteins followed by fractionation of the residual polysaccharides, the purification involves depolymerization, as is shown in Fig. 2, and the product, free of its slightly viscous impurities, has a lower viscosity (Curve VII_s) than the crude initial product (Curve VI_s). Therefore it seems impossible to obtain in the pure state products possessing a viscosity equal to that of the pleural fluid described by Meyer and Chaffee (13).

The irreversible loss of viscosity due to high temperature or esterification is compatible with the hypothesis of Ogston and Stanier (14) that the particles of hyaluronic acid are interconnected in a micelle with a loose, sponge-like structure. The sudden fall of the viscosity at a determined temperature should be due to the breaking of the interparticle links and is similar to the breakdown of the starch micelle by heat (21). The breakdown at room temperature under the action of diazomethane is evidence of the rôle of the carboxylic groups in these interconnections between particles.

Because of the slight solubility of hyaluronic acid in non-aqueous media it is very difficult to esterify hydroxyl groups and thereby obtain acetyl derivatives soluble in non-polar solvents. This difficulty is shared by other

polysaccharides containing amino sugars. All measurements of molecular weight, or of the dimensions of the molecular aggregate, can therefore be only an approximation of the average among the various aggregates of the molecules of hyaluronic acid.

The salt content of the acetates is in general below the theoretical value. It is possible that under the action of acetic anhydride an esterification between the chains takes place with formation of tridimensional polymers aggregated in micelles, which would explain the great drop in solubility. As in the case of the free hyaluronic acid esterification degrades the micelle and permits solution. It seems, however, that the greater part of the carboxylic groups cannot be reached. Either they may be engaged in ester linkage or they may be protected by the rest of the molecule. The difficulty in obtaining a more complete acetylation could also arise from these same causes.

The formation of a trityl derivative indicates that the hydroxyl group in position 6 of the glucosamine moiety is not involved in the linkage with glucuronic acid. However, the poor yield from tritylation, which is due to the general difficulties of esterification, does not allow definite conclusions to be drawn.

SUMMARY

A combination of methods for the preparation of hyaluronic acid based on enzymatic digestions and precipitations by ammonium sulfate in the presence of pyridine is described. Complete isolation of hyaluronic acid from human umbilical cord in the form of the sodium salt, possessing a very high viscosity, and in a high state of purity is effected. The free acid was obtained by passage of the sodium salt through an acid ion exchange column and its acid equivalent was found to be 395 (theoretical, 379). Starting from this acid, the sodium salt and the methyl hyaluronate were prepared. Acetylation experiments in pyridine solution yielded products containing only two to three acetyl groups per repeating unit. The formation of a derivative containing one trityl group seems to indicate that position 6 of the glucosamine moiety is not linked.

The viscosity measurements confirmed the view that hyaluronic acid consists of aggregates of filaments of high molecular weight. These measurements also showed that the aggregates rapidly depolymerized above 90° or when subjected to conditions aimed at esterifying the free carboxyl or hydroxyl groups.

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STUDIES OF ARTERIOVENOUS DIFFERENCES IN BLOOD SUGAR*

V. EFFECT OF EPINEPHRINE ON THE RATE OF GLUCOSE ASSIMILATION

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Hypoglycemia in normal individuals, as we have reported (1-3), entails a depression in the rate of peripheral glucose assimilation. This change is reflected and can be gaged by a prompt shrinkage in the difference between the glucose levels of arterial and venous blood (A-V difference), as soon as the blood sugar falls a few mg. below its normal postabsorptive level. Considerable experimental evidence indicates that this change is elicited by an insulin-antagonistic neuro-endocrine mechanism, involving the adrenal-pituitary system, which is activated by hypoglycemia. This explanation is in harmony with well known facts. Cannon and his associates (4) showed in 1924 that in rabbits epinephrine secretion is increased after insulin injection as soon as the blood sugar falls to about 80 mg. per cent. This is of the same quantitative order as the hypoglycemias in our own experiments (1). A second pertinent fact was furnished as early as 1913 by Wilenko (5), who showed that the rate of glucose assimilation in the heart muscle is greatly depressed by the subcutaneous injection of epinephrine. This finding, correlated with that of Cannon, points to epinephrine as the primary endocrine agent in the process leading to an inhibition of peripheral glucose assimilation that follows in the wake of hypoglycemia.

This concept is weakened, however, by a controversy concerning the action of epinephrine on extrahepatic glucose assimilation. Several workers presented good experimental evidence that was in harmony with Wilenko's. Thus Wiechman (6) in 1927 determined A-V differences during hyperglycemic states, produced in one group of healthy persons by the feeding of 100 gm. of glucose, in another by the subcutaneous injection of from 1 to 2 mg. of epinephrine, in the postabsorptive state. These experiments showed that, while alimentary hyperglycemia entailed a great increase in the rate of peripheral glucose assimilation, such response was virtually absent during epinephrine hyperglycemia. About the same time Cori and Cori (7) reported identical observations.

Soskin *et al.* (8, 9), on the other hand, taking exception to the experimental procedure employed by Cori and Cori, repeatedly denied that epi-

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nephrine causes a decrease in A-V differences, and stated on this basis that they "obtained no evidence that epinephrine decreases the utilization of sugar in the muscles." Yet further studies by Cori, Fisher, and Cori (10), executed with meticulously careful methods, again showed that epinephrine depressed A-V differences. But a few years later Himsworth and McNair (11), from the results of an entirely different experimental procedure, concluded that epinephrine not only fails to decrease, but actually increases, the rate of extrahepatic glucose assimilation. However, Ingle and Nezamis (12) reported recently that epinephrine inhibits glucose assimilation in eviscerated rats. In view of this situation, we attempted to procure additional experimental evidence that might contribute to the clarification of the problem.

Method

The investigators just cited, with the exception of Wiechman, made their observations on anesthetized animals, in some instances (Himsworth, Ingle) with surgical manipulations. Desiring to avoid non-physiological conditions as far as possible, we decided, first, to use healthy young persons (physicians and laboratory workers) as subjects for this study, and secondly, to avoid large doses of epinephrine (like Wiechman's 2 mg. doses), which raise blood pressure and increase heart rate and blood flow. Subcutaneous injection of 0.2 mg. of epinephrine to men weighing between 65 and 75 kilos satisfied this requirement. This choice was based on the observations of Trendelenburg (13) and of Cori *et al.* (14), who found that 0.2 mg. is the minimal dose that produces detectable effects in an adult man. Cori and his colleagues found that in man 0.2 mg. given subcutaneously exerts about the same effect as 0.00025 mg. per kilo per minute injected intravenously. In subsequent experiments (10) they observed that intravenous injection of double this dose, *i.e.* 0.03 mg. per kilo per hour, caused an increase of but 10 mm. in blood pressure. "In such a case," they stated, "one finds a decrease in the blood flow through the leg, so that the sugar uptake is actually smaller than is indicated by the arterio-venous differences."

In the light of these observations, 0.2 mg. of subcutaneous epinephrine appeared to be a safe dose, and we have ascertained that it elicited neither subjective symptoms nor any changes in the pulse rate and blood pressure in any of the subjects included in this report. However, 0.2 mg. of epinephrine did not raise the postabsorptive glycemic level to an appreciable extent. The example given in Table I shows that the maximal increase in a 68 kilo man was only 12 mg. per cent, comparable to an alimentary hyperglycemia produced by the ingestion of 5 gm. of glucose. The A-V differences, as may be noted, did not change significantly either in alimentary

or in epinephrine hyperglycemia of such slight degree. We tried therefore another experimental approach. We fed the subject 50 gm. of glucose and recorded the changes in the A-V difference during alimentary hyperglycemia. A few days later the subject went through a second test, which differed from the first only in that 0.2 mg. of epinephrine was injected about 10 minutes before the ingestion of 50 gm. of glucose. This method proved to be satisfactory; it showed conclusively the depressing effect of epinephrine on the rate of peripheral glucose assimilation.

TABLE I

Arterial and Venous Glycemic Levels in Healthy Man after Subcutaneous Injection of 0.2 Mg. of Epinephrine, and During Comparable Alimentary Hyperglycemia

Time after injection	Glucose per 100 cc. of		A-V difference
	Arterial blood*	Venous blood	
	0.2 mg. epinephrine, subcutaneously		
<i>hrs.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg. per cent</i>
0	95.3	92.1	3.2
0.5	107.7	101.8	5.9
1	107.2	103.1	4.1
2	95.0	90.5	4.5
3	92.6	89.9	2.7
4	89.9	87.5	2.4
Time after ingestion	5 gm. glucose by mouth		
<i>min.</i>			
0	91.2	90.0	1.2
15	111.5	109.3	2.2
30	102.4	101.0	1.4
45	93.8	90.2	3.6

* The arterial blood sugar levels in this study were determined in capillary (finger) blood.

Epinephrine and Peripheral Glucose Assimilation

Our subjects showed considerable individual differences in their quantitative response to the action of epinephrine, differences that can be taken for granted in the response to the action of any hormone. But such differences come properly to the fore only in carefully devised tests which permit minimal doses of hormones to act, because larger doses, as we have pointed out in the instance of insulin (2), tend to offset and obliterate individual differences of moderate degrees. In the present study, four out of six persons exhibited to 0.2 mg. of epinephrine a pronounced response of the same type, while two others were more or less resistant. The results of the tests on the four sensitive persons are recorded in Table

II and graphically presented in Fig. 1. It is evident from the data that a small dose of epinephrine sufficed to depress the rate of peripheral glucose assimilation in all four of these subjects. This fact is most conspicuous

TABLE II
Showing Inhibitory Effect of Adrenalin on Peripheral Glucose Assimilation

Time after glucose feeding	Glucose per 100 cc. of		A-V difference	Glucose per 100 cc. of		A-V difference
	Arterial blood	Venous blood		Arterial blood	Venous blood	
	hrs.	Subject 1; 50 gm. glucose by mouth			Subject 2; 50 gm. glucose by mouth	
0	87.2	85.9	1.3	83.4	79.9	3.5
0.5	133.4	126.9	6.5	167.4	135.0	32.4
1	146.3	125.6	20.7	159.3	131.0	28.3
2	105.8	89.6	16.2	124.5	102.6	21.9
3	73.7	66.4	7.3	70.7	67.0	3.7
3.5	83.2	82.0	1.2	76.4	75.1	1.3
	0.2 mg. epinephrine subcutaneously, 10 min. before glucose feeding			0.2 mg. epinephrine subcutaneously, 10 min. before glucose feeding		
0	99.7	96.7	3.0	87.3	84.0	3.3
0.5	174.7	172.3	2.4	193.3	186.3	7.0
1	211.1	209.5	1.6	251.9	246.8	5.1
2	112.3	108.8	3.5	171.2	170.1	1.1
3	131.5	124.5	7.0	48.6	47.5	1.1
3.5	62.9	61.0	1.9	68.6	67.5	1.1
	Subject 3; 50 gm. glucose by mouth			Subject 4; 50 gm. glucose by mouth		
0	90.2	88.8	1.4	101.0	99.6	1.4
0.5	145.2	127.7	16.5	164.7	149.0	15.7
1	140.4	124.7	15.7	145.0	139.9	5.1
2	115.0	101.8	14.2	111.3	109.4	1.9
3	72.9	69.1	3.8	84.2	83.2	1.0
3.5	72.9	70.2	2.7			
	0.2 mg. epinephrine subcutaneously, 10 min. before glucose feeding			0.2 mg. epinephrine subcutaneously, 10 min. before glucose feeding		
0	94.5	93.2	1.3	100.4	97.2	3.2
0.5	176.9	172.5	4.4	169.6	164.1	5.2
1	194.9	186.8	8.1	224.1	216.8	7.3
2	140.9	131.5	9.4	172.0	170.6	1.4
3	78.3	77.0	1.3	108.0	101.3	6.7
3.5	75.6	74.3	1.3	82.4	77.8	4.6

in Subject 2, whose A-V difference increased to a maximum of 32.4 mg. per cent as the result of glucose feeding, but reached only 7.0 mg. per cent when epinephrine injection preceded the ingestion of glucose.

Closer scrutiny of our data reveals several other pieces of information. Thus they show that the changes in the glycemic level always represent a dynamic balance of the mutually antagonistic actions of epinephrine in one direction, and of insulin in the opposite. It may be seen in Table II that the blood sugar rose to much higher peaks and then dropped much more steeply in the epinephrine tests than in the simple glucose feeding tests. This phenomenon was most marked in Subject 2, whose blood sugar (arterial) fell from a peak of 167.4 to a low of 70.7 mg. per cent in the course of 2½ hours after glucose feeding, whereas in the test with epinephrine the blood sugar rose to the much higher peak of 251.9 mg. per cent

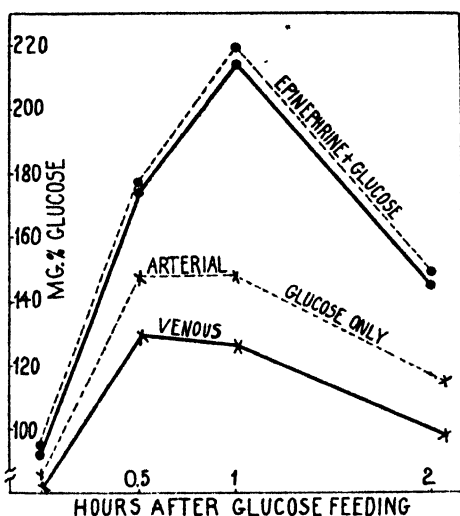


FIG. 1. Composite curves showing the arterial (capillary) and venous blood sugar curves after the ingestion of 50 gm. of glucose, with and without the simultaneous action of 0.2 mg. of epinephrine injected subcutaneously. The decrease of the arteriovenous differences under epinephrine action is conspicuously shown by the curves.

and then dropped to the much lower level of 48.6 mg. per cent within 2 hours.

From an analysis of these changes one can arrive at a rough estimate as to the duration of epinephrine action in these experiments. The data for Subject 2 present a good case in point. It may be noted that after glucose feeding alone the blood sugar had reached its peak of 167.4 mg. per cent in 30 minutes, and then began to fall during the second 30 minutes, indicating that the insulin produced by hyperglycemia began to show its effects at this time. In the epinephrine test, on the contrary, the hyperglycemia, instead of declining, continued to rise during the second 30 minutes and

attained a peak of 251.9 mg. per cent, despite the fact that in this instance the available insulin supply, owing to the higher glycemic levels, was probably greater than in the first test. Epinephrine action had simply overwhelmed and practically obliterated insulin action. During the 2nd hour in the epinephrine test the hyperglycemia, 171.2 mg. per cent, was still on a level that was higher than the peak in the test with glucose feeding alone, indicating that epinephrine was still in action during the 2nd hour after injection. This inference is strengthened by the fact that the A-V difference during this high hyperglycemia was as low as 1.1 mg. per cent, whereas with glucose alone, at the corresponding time interval it was as high as 21.9 mg. per cent at the moderate hyperglycemic level of 124.5 mg. per cent. After the 2nd hour, epinephrine action apparently ceased and insulin action became predominant, as shown by the precipitate fall of the blood sugar to the low level of 47.5 mg. per cent.

It is hardly conceivable that the insulin supply that caused this hypoglycemia at the end of the 3rd hour should have suddenly emerged in the course of the 3rd hour, when the blood sugar had already been falling steeply. For all we know, insulin secretion was stimulated during the preceding hyperglycemic interval. The exaggerated hyperglycemia and the abnormally small A-V difference that went with it do not mean that the insulin supply was not increased (as it normally is) under the influence of hyperglycemia; they only show that during the 2nd hour after injection the epinephrine still actively opposed and stifled the action of insulin. Then, during the 3rd hour, pronounced hypoglycemia was evident, as a sign of definite insulin action. Whether or not epinephrine action at this time had been completely dissipated, or whether it still was there but so feeble that insulin action had simply overwhelmed it, is too fine a detail to be answered by our experiments. The view that insulin secretion is stimulated by hyperglycemia, irrespective of its being produced by epinephrine, is in line with the known fact that epinephrine injection entails a marked fall in the potassium concentration of blood plasma, a well recognized response to insulin action.

It may be said that the finer details of our findings cannot be properly understood unless interpreted as the results of a dynamically shifting balance between the mutually opposing actions of epinephrine and insulin. So interpreted, they permit the inference that the action of 0.2 mg. of subcutaneous epinephrine in man is spread over approximately 2 hours, which would be the equivalent of an average rate of $0.2/120 = 0.00167$ mg. per minute, or in a 70 kilo man 0.000024 mg. per kilo per minute. Such a dose, as pointed out before, is far below the amounts which are apt to change blood pressure or the rate of blood flow.

It was stated before that two of our six subjects exhibited distinct

resistance to epinephrine action. For comparison with the preceding four cases the results obtained on the most resistant subject are presented in Table III. As may be seen, 0.2 mg. of epinephrine failed to depress peripheral glucose assimilation; in fact, the A-V differences are suggestive of a slight increase. It also may be noted that hyperglycemia stayed within the normal range; in other words, the glucose tolerance remained normal although somewhat poorer than in the test with glucose alone. In the

TABLE III
Subject Resistant to Epinephrine Action

Time after glucose	Glucose per 100 cc. of		A-V difference
	Arterial blood	Venous blood	
50 gm. glucose by mouth			
hrs.	mg.	mg.	mg. per cent
0	87.2	85.9	1.3
0.5	133.4	126.9	6.5
1	146.3	125.6	20.7
2	105.8	89.6	16.2
3	73.7	66.4	7.3
4	83.2	82.0	1.2
0.2 mg. epinephrine subcutaneously, 10 min. before glucose feeding			
0	89.9	85.3	4.6
0.5	156.6	146.1	10.5
1	174.2	145.8	29.4
2	88.0	82.6	5.4
3	78.3	76.7	1.6
0.3 mg. epinephrine subcutaneously, 10 min. before glucose feeding			
0	87.7	86.9	0.8
0.5	209.2	204.4	4.8
1	238.7	219.1	19.6
2	72.9	60.9	12.0
3	70.2	67.2	3.0

second test with epinephrine, in which the dose was raised to 0.3 mg., hyperglycemia rose to diabetic levels, and the A-V differences were a little smaller than with glucose alone.

The data in Table III, however, are not entirely satisfactory for the evaluation of the differences between the rates of peripheral assimilation in the three tests performed on this subject. We have shown in an earlier paper (15) that in any given individual a rough proportionality obtains

between the rate of peripheral glucose assimilation and the height of hyperglycemia; i.e., that higher hyperglycemic levels entail higher A-V differences. Since in the tests involving epinephrine injection the hyperglycemic levels were increased to a variable extent, the corresponding changes in A-V differences reflect the influence of two factors: that of epinephrine action and that of augmented hyperglycemia. Hence, if the action of epinephrine alone is to be gaged, the influence of the variable hyperglycemias must be eliminated from the picture. This can be accomplished by introducing a common correlative (denominator) into the ratio of peripheral assimilation to hyperglycemia. The numerator of this ratio is the sum of the A-V differences in the course of an observation, denoted as *assimilation index*; the denominator (correlative) is the *hyperglycemic sum*, which represents the sum of the increments in the arterial blood sugar above the fasting level during the corresponding time interval. When 100 is used as the common correlative, the expression (assimilation index)/(hyperglycemic sum) $\times 100$ is obtained and is termed the *relative assimilation index*. This figure shows what the assimilation index in each test would be if the extent of the hyperglycemia were identical in all of them. Hence the relative assimilation index represents with fair approximation the influence of epinephrine action, independently of the variations in the hyperglycemic states.

In Table IV are recorded the data obtained in this manner not only for the two resistant subjects, but also for the four subjects included in Table II. We also added a test with 0.5 mg. of epinephrine; this was carried out in a clinical diagnostic procedure outside the scope of the present study, but the results appear sufficiently interesting to be included in this series. It may be noted that in the first four subjects the uncorrected assimilation index itself shows conclusively enough the strong depression of the peripheral glucose assimilation under the influence of epinephrine action. Yet the actual effect is greater than thus indicated. In Subject 1, for instance, the uncorrected assimilation index shows that the rate of assimilation was lowered by epinephrine to about one-sixth of the normal rate (7.5 as against 43.5), whereas according to the relative assimilation index it was decreased more nearly to one-tenth (3.8 as against 35.0). And this relationship is of the same order for Subjects 2, 3, and 4.

The meaning and value of the relative assimilation index are far more evident in the evaluation of the data concerning the epinephrine-resistant Subjects 5 and 6. Subject 5, for instance, showed no inhibition at all in the peripheral glucose assimilation under the influence of 0.2 mg. of epinephrine, when it is judged on the basis of the uncorrected assimilation index. The true picture, however, as represented by the relative assimilation index, shows a decrease of 14 per cent (a fall from 35.0 to 30.0). Like-

wise, the action of 0.3 mg. of epinephrine, according to the uncorrected assimilation index, apparently caused only a mild depression of 16 per cent (a fall from 43.4 to 36.4), whereas the change, as more truly reflected in the relative assimilation index, was 62 per cent (a fall from 35.0 to 13.3).

Epinephrine and Hepatic Glycogenolysis

Ever since the discovery of "adrenalin diabetes" 49 years ago, it has been taken for granted that epinephrine causes hyperglycemia and glycosuria by accelerating hepatic glycogenolysis. What is the basis of this assump-

TABLE IV

Changes in Rate of Peripheral Glucose Assimilation As Shown by Relative Assimilation Index (RAI)

Subject No.	Dose of epinephrine (sub-cutaneous)	0.5 hr.		1 hr.		2 hrs.		Hyper-glycemic sum	Assimilation index	RAI
		Increment*	A-V difference	Increment	A-V difference	Increment*	A-V difference			
		mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent			
1	0	46.2	6.5	59.1	20.7	18.6	16.2	123.9	43.4	35.0
	0.3	75.0	2.4	111.4	1.6	12.6	3.5	198.0	7.5	3.8
2	0	84.0	32.4	75.9	28.3	41.1	21.9	200.0	82.6	41.2
	0.2	106.0	7.0	164.6	5.1	83.9	1.1	354.5	13.2	3.7
3	0	55.0	16.5	50.2	15.7	24.8	14.2	130.0	46.4	35.7
	0.2	82.4	4.4	100.4	8.1	46.4	9.4	229.2	21.9	9.6
4	0	63.7	15.7	44.0	5.1	10.3	1.9	118.0	22.7	19.3
	0.2	69.2	5.2	123.7	7.3	71.6	1.4	264.5	13.9	5.3
5	0	46.2	6.5	59.1	20.7	18.6	16.2	123.9	43.4	35.0
	0.2	66.7	10.5	84.3	29.4	0	5.4	141.0	45.3	30.0
6	0.3	121.5	4.8	151.0	19.6	0	12.0	272.5	36.4	13.3
	0	77.4	19.1	82.6	38.9	10.8	3.5	170.8	61.5	36.0
7	0.2	133.1	7.0	163.6	34.3	21.9	12.5	318.6	53.8	16.9
	0.5	93.9	2.1	141.5	1.7	25.4	1.7	260.8	5.5	2.1

* Increment of arterial blood sugar above fasting level.

tion? As an answer to this question we quote from Peters and Van Slyke (16): "It was early discovered that the administration of epinephrine caused the blood sugar to rise rapidly, often high enough to provoke glycosuria. The rise obviously results from accelerated hepatic glycogenolysis, since muscle glycogen can not be mobilized into the blood as glucose. Moreover, adrenalin hyperglycemia does not occur when the liver and its glycogen supply are cut out of the circulation." The facts, as stated in this quotation, are unimpeachable, but not the interpretation of the facts. We submit here a different concept which is in complete harmony with the observed facts, and hence capable of explaining them no less satisfactorily

than the generally accepted concept. Our interpretation is not a substitute for the current one; rather it should modify and complement it.

The defect in the current reasoning is that it overlooks the fact that the rate of flow of glucose in the liver cell can be affected at two points instead of only one; i.e., that it is not the rate of glycogenolysis alone that is subject to variations, but that *the rate of glucose assimilation* also can change. Thus it is evident that a rise in the blood sugar level (in the postabsorptive state) does not necessarily imply an increase in the rate of hepatic glycogenolysis, but may just as well be the result of a decrease in the rate of glucose assimilation. Every known piece of experimental evidence (obtained

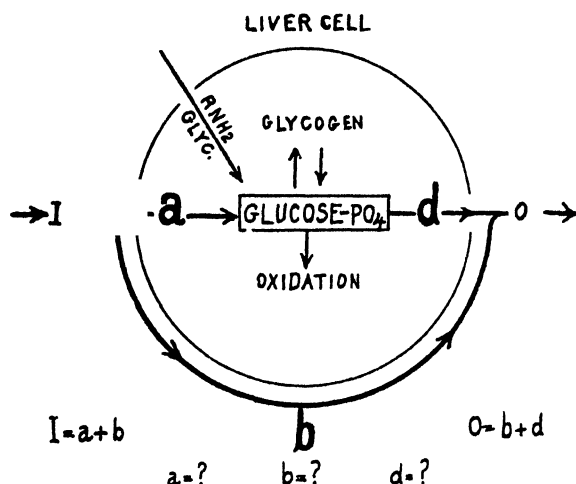


FIG. 2. Diagram of the flow of glucose in the liver cell, illustrating that the glucose output from the liver (O) represents the sum of unassimilated glucose (b) plus the glucose dissimilated and excreted by the liver cell (d), and that in consequence an increase in glucose output (O) does not necessarily reflect an increase in the rate of glycogenolysis; this may just as well be ascribed to a decrease in the rate of assimilation (a).

in the intact animal) which is supposed to prove unequivocally that hyperglycemia is the result of accelerated hepatic glycogenolysis, can, we submit, prove with equal validity the view that the increase in the glycemic level resulted from a decrease in the rate of hepatic glucose assimilation.

In Fig. 2 is presented a diagram for the elucidation of our thesis. I represents the amount of glucose carried to the liver by way of its afferent vessels (often denoted in the literature as the glucose "intake"); O is the glucose carried from the liver in the efferent vessels (the glucose "output"). These are the two quantities capable of quantitative determination *in the intact animal*. An increase in O is traditionally regarded as an obvious

sign of an increase in the rate of glycogenolysis. A glance at Fig. 2 shows, however, that, while this might be the case, the known quantities, I and O , do not yield the information required to prove it. I , as the diagram indicates, branches off into two fractions: one of these, a , is the fraction that is assimilated by the liver (*i.e.*, is transferred from the blood into the cell, via phosphorylation), while b , the unassimilated fraction, by-passes the cell and leaves the liver unchanged. The other known quantity, O , then, represents the sum of two glucose fractions: one is the amount dissimilated and secreted by the liver cell, d , to which is added b , the unassimilated fraction of I . The situation, then, is that the experimental measurements supply two equations: $I = a + b$, and $O = b + d$. These two equations comprise three unknown quantities, with the consequence that determination of I and O yields no information as regards changes either in the rate of glycogenolysis or in the rate of assimilation. The available experimental data represent only variations in the dynamically shifting quantitative relationship between the rates of the two opposite processes.

In the light of this concept it may be stated that there is no experimental evidence to prove that epinephrine increases d , the rate of hepatic glycogenolysis, since a decrease in the rate of hepatic glucose assimilation alone suffices to account for an accumulation of glucose in the blood. In view of the fact that glucose assimilation in the liver is managed essentially by the same enzyme system as in the peripheral tissues, the inference is virtually inescapable that *a factor which inhibits glucose assimilation in the peripheral tissues exerts the same influence also in the liver*. Thus depression of glucose assimilation in both liver and peripheral tissues suffices to account for epinephrine hyperglycemia, without requiring an acceleration of hepatic glycogenolysis (d). This is not to say that d actually escapes any change during epinephrine action. We only state that the contrary cannot be proved in the intact animal (in the liver *in situ*); only in experiments with the isolated liver or with liver slices, in which a can be eliminated by reducing I to zero, is it possible to determine changes in the rate of hepatic glucose dissimilation.

DISCUSSION

The studies reported in this paper furnish, we believe, clear cut results which demonstrate the fact that epinephrine strongly inhibits the rate of peripheral glucose assimilation.¹ This is in line with the earlier findings

¹ Discussion of the mechanism and pathways of epinephrine action is outside the scope of this paper. It may be in place, however, to point out that Wilenko (5) had recognized as early as 1913 "that adrenalin acts indirectly, namely, by means of affecting another organ." This observation was eventually substantiated by the

of Wilenko, of Wiechman, and of Cori and his collaborators. In regard to the conflicting findings of Soskin and his colleagues, they admitted the validity of the objections raised against the non-physiologic procedures they employed in their first study. "The insertion of glass and rubber tubing into the circulatory system and the continued heparinization of amyralized animals led to the gradual deterioration of their condition and of the circulation of blood," they conceded in a report of a second study (9) in which they had modified the experimental conditions. But the results of their second set of experiments still seem to us entirely unconvincing. Although they substantially lowered the rate of epinephrine infusion, they still used from 4 to 8 times as large doses as Cori *et al.*; secondly, we find rather unconvincing the experimental data which indicated (and led their authors to conclude) that free glucose readily passes in and out of muscle cells, often giving rise to much higher glucose levels in the venous than in the arterial blood (inverted A-V differences). The validity of such data is the more questionable as Cori and his coworkers never found inverted A-V differences in their own experiments which were conducted along similar lines (intravenous epinephrine injections in amyralized dogs). It cannot be readily determined whether or not Soskin's results were distorted by excessive epinephrine doses (40 times as great as were used in our own experiments) or by some other technical factors, such as for instance those which may vitiate A-V difference determinations (10, 15), or by both.

As to Himsworth's report, which claims that epinephrine increases the rate of peripheral glucose assimilation instead of decreasing it, we were surprised to find that this author had drawn conclusions which diametrically contradicted his own experimental data. The experiments were carried out on rabbits in which the liver was excluded from circulation, with the consequence that the blood sugar level gradually declined (as it does in animals after liver extirpation). The rate at which glucose under this condition disappears from the blood evidently reflects the rate of extra-hepatic assimilation. When intravenous epinephrine was given to such animals, the blood glucose suddenly (in less than 2.5 minutes) dropped by about 10 mg. per cent. From that point on during the ensuing 2 hours, however, the sugar curve ran parallel with that of the rabbits which were

work of numerous investigators, who identified the hypophysis as "another organ" by means of which epinephrine action affects the glycemic level and the flow of glucose between cells and extracellular fluids. The extensive work of C. N. H. Long and his associates in this field is generally known. It is to be noted, however, that epinephrine can produce hyperglycemia also independently of the hypophysis, but that this effect is considerably greater in the intact than in the hypophysectomized animal (17, 18).

not given epinephrine. At this time the blood sugar in the epinephrine-treated animals began to rise and, within a few minutes, increased by about 10 mg. per cent. In other words, the very same amount of sugar that had suddenly disappeared after the injection of epinephrine reappeared 2 hours later, about the time when epinephrine action ceased. Explanation of this phenomenon is irrelevant from the point of view of our present considerations; the relevant and pertinent fact is that for the important interval between the vanishing and the return of 10 mg. per cent of blood sugar, the rate of peripheral glucose assimilation was by no means increased (it even showed occasional decrease) by the action of injected epinephrine. It is also of interest to note that Himsworth gave his 2 kilo rabbits 0.25 mg. of intravenous epinephrine in a single injection; this is a very large dose indeed, considering that it corresponds to a 9 mg. dose for a 70 kilo man. The impact of such doses is apt to elicit all sorts of violent reactions, especially in animals which, owing to the inactivation of their livers, had already been exposed to non-physiologic conditions and to considerable stress.

SUMMARY

1. Experiments described in this paper clearly show that epinephrine action strongly depresses the rate of peripheral glucose assimilation. Close scrutiny of the pertinent controversial literature persuaded this author that conclusions to the contrary were not supported by satisfactory experimental evidence.

2. Available observations on intact animals furnish no basis for the currently accepted concept that the hyperglycemic response to epinephrine action is an obvious result of accelerated hepatic glycogenolysis. While such a response may (and even is likely to) take place, depression of the rate of glucose assimilation in both the liver and in the peripheral tissues, which is caused by epinephrine, can readily account for a rise of the glycemic level, without requiring any acceleration of the hepatic glycogenolysis.

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LIPOGENESIS AND GLUCOSE OXIDATION IN THE LIVER OF THE ALLOXAN-DIABETIC RAT*

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Stetten and his associates showed, with the aid of deuterium, that the rat rendered diabetic by the injection of alloxan loses to a great extent its ability to utilize glucose for the synthesis of fatty acids (1). Since the liver is probably the principal site for lipogenesis (2), we have studied the conversion of C¹⁴-labeled glucose to fatty acids by liver slices of alloxan-diabetic rats. It is shown here that utilization of carbohydrate for fatty acid formation is drastically reduced in the diabetic liver.

EXPERIMENTAL

Diets—Rats of the Long-Evans strain were used throughout. They were fed either the stock diet or Diet S. The former contained 68.5 per cent whole wheat, 5 per cent casein, 10 per cent fish-meal, 1.5 per cent salt, 5 per cent fish oil, and 10 per cent alfalfa. This diet contained approximately 50 per cent carbohydrate and 22 per cent protein.

Diet S, also high in carbohydrate, had the following composition: dextrose 60 per cent, casein (Labco, vitamin-free) 22 per cent, brewers' yeast 6 per cent; salt mixture (3) 6 per cent, and Cellu flour 6 per cent. The rats fed this diet received vitamins A and D in the form of a fish oil which contained 400 A. O. A. C. chick units of the former and 3000 U. S. P. units of the latter per cc.

Diabetic Rats—Diabetes was induced by a single intravenous injection of a 5 per cent solution of alloxan monohydrate (50 mg. per kilo, body weight). The rats that showed a marked polyuria were placed in individual metabolism cages and their food and water intake, urine volume, and glucose excretion were measured daily. During this preliminary diagnostic period, the rats were fed the stock diet which was supplemented on alternate days by an adequate vitamin mixture (4).

Urine was collected daily and its glucose content was determined by oxidation with potassium ferricyanide and subsequent titration with ceric sulfate (5). Blood sugar on rats in the fed and fasted state was determined

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at repeated intervals, about 0.5 cc. of blood for this purpose being taken from the tail vein. The blood was deproteinized with ZnSO_4 and NaOH according to Somogyi (6), and the glucose in the filtrate was determined as described above.

Only rats that manifested polyuria and glycosuria for 2 months or longer were employed in these experiments. While fasting, the diabetic rats used in this study had, with few exceptions, blood sugar levels in excess of 200 mg. per cent. The diabetic histories of the rats are shown in Table I.

Radioactive Glucose—The radioactive glucose used was made photosynthetically as described by Putman *et al.* (7). Its carbons were uniformly

TABLE I
History of Alloxan-Diabetic Rats in This Study

Rat No.	Sex	Rat weight		Duration of diabetes	Daily food intake*	Degree of diabetes			
		When injected	When sacrificed			Range, urine flow in 24 hrs.†	Maximum urine sugar for 24 hr. sample†	Fasting blood sugar‡	Blood sugar at time of sacrifice (not fasting)‡
		gm.	gm.	days	gm.	cc.	gm.	mg. per cent	mg. per cent
D1§	M.	224	270	135	27	55-105	6.7	232	525
D2§	"	96	125	135	18	25- 50	3.4	400	455
D3§	"	100	160	160	30	35- 90	5.2	254	538
D4	"	270	305	100	24	30- 74	5.6	205	588
D5	"	120	160	125	25	50- 90	6.0	330	454
D6	"	140	250	125	30	50-100	8.3	292	
D7§	"	130	175	125	28	70-130	5.8	250	
D8	F.	152	235	105	23	40- 80	8.0	184	411
D9	"	137	190	105	32	50-110	10.7	165	432
D10	"	122	144	90	21	60-110	7.7	191	403

* Average for amounts ingested daily during 2 weeks before sacrifice.

† Determined 1 to 3 weeks before sacrifice.

‡ Whole blood was used for sugar determinations.

§ Fed Diet S for 2 or more weeks. All the others were fed the stock diet.

labeled with C^{14} . We are indebted to Mr. S. Abraham for its preparation. The activity of the glucose used in each experiment was approximately 10,000 c.p.m. per mg. of glucose. This was determined by oxidizing the glucose to CO_2 and measuring the activity of the latter in the form of BaCO_3 (8).

Preparation of Slices—The rats were sacrificed by a blow on the head and their livers were rapidly removed and placed in cold oxygenated Ringer-bicarbonate buffer (9). Slices were prepared free-hand by means of a thin razor blade and collected in a Petri dish containing the same buffer. Portions (approximately 500 mg.) were gently blotted on a moist filter paper, weighed on a torsion balance, and then transferred to the incubation flasks.

Determination of Carbohydrate Content of Liver Slices before Incubation—

A separate portion of liver slices taken directly from the Petri dish was used for the determination of the carbohydrate content. A weighed portion (approximately 500 mg.) was macerated and heated with 5 cc. of 1 N H_2SO_4 for 3 hours on a steam bath. The mixture was filtered, the residue on the filter paper was thoroughly washed with hot water, and the combined filtrates and washings were made to volume. Aliquots were first neutralized with Na_2CO_3 , then treated with $ZnSO_4$ and $NaOH$ according to the method of Somogyi (6), and the clear supernatant obtained was analyzed for glucose (5).

*Incubation Procedure—*The slices were incubated in a specially designed flask described by Chernick *et al.* (10). The medium used was a Ringer-bicarbonate buffer (9) to which glucose was added. The incubation period was 3 hours. The details of the procedure used in this study have been recorded elsewhere (10).

*Collection and Determination of $C^{14}O_2$ Evolved—*The flask contained a center well into which was inserted a roll of filter paper. At the end of the experiment, the CO_2 evolved was collected on the filter paper by injecting a KOH solution into the center well. The radioactivity of the CO_2 was determined after the manner of Entenman *et al.* (8).

*Determination of Fatty Acids—*The contents of the main compartment of the incubation flask were filtered through No. 1 Whatman filter paper. The slices and other residue on the filter paper were washed several times with small amounts of distilled water. The slices and other residue that remained on the filter paper were transferred to a flask containing 10 cc. of 50 per cent alcohol and 1 cc. of 90 per cent KOH . The mixture was heated on a steam bath for 2 hours. The modification of the method of Sperry *et al.* (11), used for the isolation of fatty acids from this hydrolysate, has been described elsewhere (10). The fatty acids were finally obtained as a solution in petroleum ether. The fatty acid content of this solution was determined by a modification of Bloor's oxidative procedure (12). The C^{14} content of the fatty acids was measured by the direct mount technique described by Entenman *et al.* (8).

In several experiments, with both normal and diabetic liver slices, cholesterol was isolated as the digitonide and found to contain a negligible number of C^{14} counts.

Results

Conversion of Added Glucose- C^{14} to Fatty Acids and CO_2 by Liver Slices of Normal and Alloxan-Diabetic Rats Suspended in Glucose Concentration of 400 Mg. Per Cent

The results of experiments carried out with liver slices prepared from normal and alloxan-diabetic rats are recorded in Table II. Both types of

rats were fed high carbohydrate diets before they were sacrificed. In view of the effects of fasting upon lipogenesis reported elsewhere (13), care was taken to insure that these rats consumed adequate amounts of food until sacrificed. Postmortem examination confirmed the presence of considerable amounts of food in the stomach.

TABLE II

Conversion of C¹⁴-Glucose to Fatty Acid by Normal and Diabetic Liver Slices

Each incubation flask contained about 500 mg. of liver slices and 5 cc. of Ringer-bicarbonate buffer. Two such flasks were prepared from the liver of each rat. Incubated at 37.5° for 3 hours. The specific activity of glucose in the medium before the addition of slices was the same in all flasks.

Rat No.	State and body weight	Liver slices contained in 2 flasks			Glucose concentration in medium†	Added C ¹⁴ recovered per gm. liver in form of	
		Amount wet weight	Fatty acids*	Total carbohydrate†		CO ₂	Fatty acids
	gm.	mg.	per cent	per cent	mg per cent	per cent	per cent
N1§	Normal (230)	905	2.4	3.5	382	3.4	0.60
N2§	" (200)	930	2.8	3.0	382	4.7	0.77
N3	" (200)	990	3.0	4.7	396	4.5	0.81
N4	" (180)	1024	3.3	4.2	396	5.1	0.61
D1§	Diabetic	911	2.3	3.5	396	1.9	0.02
D2§	"	880	2.3	1.8	396	0.9	0.01
D3§	"	905	1.7	2.4	410	1.2	0.00
D4	"	1000	5.7	4.6	438	1.5	0.02
D5	"	1025	4.2	3.2	438	1.1	0.02
D6	"	1030	5.7	3.8	438	1.3	0.02
D7§	"	1005	3.7		418	1.2	0.00

* Determined on the whole flask contents at the end of the experiment.

† The values represent the carbohydrate content of the slices at the start of the incubation period.

‡ Before addition of slices.

§ Fed Diet S for 2 or more weeks. All the others were fed the stock diet.

|| The weights of the diabetic rats are recorded in Table I.

In all the experiments, the C¹⁴-labeled glucose was added to the medium in which the liver slices were incubated and the results are expressed as percentages of this C¹⁴ recovered as fatty acids and as CO₂.

In the four experiments with *normal* liver slices, 3 to 5 per cent of the added C¹⁴ was recovered as CO₂, and 0.6 to 0.8 per cent as fatty acids. These values are in good agreement with those reported earlier for normal rats fed high carbohydrate diets (10, 13).

Seven experiments were carried out with liver slices prepared from diabetic rats. As shown in Table I, diabetes had been present in these rats

for at least 90 days before they were sacrificed, and the blood sugar, while they were fasting, was in the neighborhood of 200 mg. per cent or higher. These rats also exhibited the usual manifestations associated with diabetes; namely, polyuria, glycosuria, etc.

The $C^{14}O_2$ produced by the diabetic liver slices was much less than normal (Table II). These slices, moreover, appeared to have lost almost completely their capacity to convert the C^{14} -glucose to fatty acids.

None of the diabetic livers studied in the experiment described above or in those to be described below can be considered fatty. The total fatty acid content of the normal livers was about 3 per cent and did not exceed 5.7 per cent in the diabetic.

Effect of Glucose Concentration in Medium on Conversion of Glucose- C^{14} to Fatty Acid and CO_2

In the preceding experiments, the concentration of the labeled glucose in the incubation medium, before the addition of the slices, was about 400 mg. per cent. In the experiments described in Table III, the concentration of the added glucose was varied from 100 to 800 mg. per cent *but the specific activity of this added glucose was the same in all the flasks*. Slices of *each* normal and of *each* diabetic liver were tested at two glucose levels: 100 and 400 in Experiment 1, 200 and 800 in Experiment 2, and 100 and 800 mg. per cent in Experiment 3. In the last two columns are recorded the C^{14} counts recovered in fatty acids and CO_2 .

An examination of the data presented in Table III shows that the $C^{14}O_2$ and fatty acid- C^{14} recoveries were depressed, in the experiments carried out with the diabetic livers, not only when the initial concentration of glucose in the medium was 400 mg. per cent but also when it was 100, 200, and 800.

In each experiment, the C^{14} recovered as CO_2 was greater at the higher glucose concentration. This was true for both diabetic and normal livers. This finding may indicate that the liver slices oxidized more glucose to CO_2 when exposed to the higher glucose concentration. On the other hand, this result may merely reflect differences in the dilution of the added glucose by the carbohydrate of the slices. One would expect that the higher the concentration of the *added* glucose in the medium, the less would be its dilution by the carbohydrate of the slices.

DISCUSSION

As much as 0.8 per cent of the glucose- C^{14} was recovered as fatty acids when *normal* liver slices were incubated for 3 hours in a medium containing 400 mg. per cent of glucose. Under identical conditions, livers of rats in which diabetes had existed for about 2 months lost almost completely the

capacity to convert glucose to fatty acids. This loss was observed not only when the glucose concentration in the medium was low (100 mg. per cent) but also when it was high (800 mg. per cent). Since large amounts of fatty acids were present in the liver slices at the end of the experiment, an increased oxidation of fatty acids in the diabetic liver slices could not account for the low fatty acid- C^{14} recoveries observed unless it is assumed that the newly formed fatty acids did not mix with the fatty acid pool in the slice, but were preferentially oxidized to such an extent that almost all of the fatty acids formed from glucose during the period of incubation were oxidized before the end of the experiment. In view of the improbability of such an assumption, we must conclude that the utilization of carbohydrate for fatty acid formation is drastically reduced in the diabetic liver.

The $C^{14}O_2$ recoveries were also depressed in the experiments with the diabetic liver slices, regardless of the concentration of the glucose added to the medium. While there can be no doubt that the diabetic liver slices converted less of the *added* glucose to CO_2 than did the normal liver slices, it cannot be concluded merely from the $C^{14}O_2$ recoveries that the total amount of glucose oxidized in the diabetic liver is depressed. The factors that influence $C^{14}O_2$ recoveries in experiments of the type carried out here were considered in some detail elsewhere (13) and it was pointed out there that dilution of the added labeled glucose by the carbohydrate present inside the slice is of considerable importance in this connection. Little or no difference in the carbohydrate contents of normal and diabetic livers was found (see Tables II and III). Since similar differences were observed between the diabetic and normal liver slices at four glucose concentrations in the medium, namely, 100, 200, 400, and 800 mg. per cent (see Table III), it would appear that the depressed $C^{14}O_2$ recoveries observed in the experiments with diabetic livers are not the result of simple dilution of the *added* glucose by the tissue carbohydrate.

It is clear that glucose utilization in the diabetic liver is impaired. But the impairment in glucose utilization for lipogenesis is far more striking than that involving the conversion of the added glucose to CO_2 . The results of the present study thus afford ample confirmation of the conclusion drawn by Stetten and Boxer (1); namely, that the failure to utilize glucose for fatty acid synthesis is a major metabolic defect in diabetes.

Previous work from this laboratory gave no indication that the over-all capacity of the alloxan-diabetic rat to convert plasma glucose to CO_2 differed significantly from that in the normal rat (14). The difference between the results obtained in the experiments *in vivo* and *in vitro* is not to be explained by variations in the degree of diabetes of the rats used in the two types of experiments, for in both the diabetes was of long standing and the rats used manifested, while fasting, blood sugars in excess of 200 mg.

per cent. The results of the two types of experiments can be reconciled by assuming that glucose oxidation remains normal or near normal in some

TABLE III

Effect of Glucose Concentration on Conversion of Glucose to CO₂ and Fatty Acids

Slices for four incubation flasks were prepared from the liver of each rat. Two of these incubation flasks contained a low concentration of glucose (100 or 200 mg. per cent), whereas the other two contained higher concentrations (400 or 800 mg. per cent). Each flask contained about 500 mg. of liver slices and 5 cc. of Ringer-bicarbonate buffer. They were incubated at 37.5° for 3 hours. Each of the four flasks was analyzed separately and the sum of the values for the two flasks at each glucose concentration is recorded. The specific activity of the glucose in the medium before addition of the slices was the same in all the flasks.

Experiment No.	Normal liver slices				Diabetic liver slices				Labeled glucose added to medium		Condition of slice	C ¹⁴ recovered per gm. liver in the form of	
	Rat No.*	Amount added to 2 flasks	Total carbo-hydrate†	Total fatty acid‡	Rat No.§	Amount added to 2 flasks	Total carbo-hydrate†	Total fatty acid‡	Amount per 2 flasks	Concentration		CO ₂	Fatty acids
		mg.	mg. per gm.	per cent		mg.	mg. per gm.	per cent	mg.	mg. per cent		c.p.m.	c.p.m.
1	N5	1010	38	3.4	D8	1030	54	2.2	10	100	Normal	2,700	105
											Diabetic	1,050	17
		1020				1020			40	400	Normal	7,350	775
											Diabetic	1,900	25
2	N6	1000	52	1.6	D9	1000	44	2.4	20	200	Normal	4,930	260
											Diabetic	1,440	10
		1000				1010			80	800	Normal	11,500	1000
											Diabetic	2,175	18
3	N7	1020	45	3.6	D10	980	39	2.0	10	100	Normal	2,550	160
											Diabetic	910	0
		990				1000			80	800	Normal	10,100	800
											Diabetic	2,650	0

* Rat N5 weighed 188 gm., Rat N6 220 gm., Rat N7 180 gm.

† The values given represent the carbohydrate content of the slices at the start of the incubation period.

‡ Determined on the whole flask contents at the end of the run.

§ The weights of the diabetic rats are recorded in Table I.

extrahepatic tissue or tissues of sufficient bulk to mask the depression observed here for the liver.

SUMMARY

1. The conversion of C¹⁴-labeled glucose to fatty acids and CO₂ was studied in surviving liver slices prepared from normal and alloxan-diabetic

rats. At the time the latter were sacrificed, diabetes had been present for 90 to 165 days and the blood sugars, while fasting, were in the neighborhood of 200 mg. per cent or higher.

2. Normal liver slices converted as much as 0.8 per cent of the added glucose- C^{14} to fatty acids when the concentration of glucose added to the medium was 400 mg. per cent. The C^{14} so recovered represented about 18 per cent of that converted to CO_2 .

3. Under identical conditions, the diabetic liver failed almost completely to convert glucose to fatty acids.

4. Oxidation of the added glucose to CO_2 in the diabetic liver was also depressed.

5. The failure by the diabetic liver, in lipogenesis and in oxidation of the added glucose to CO_2 , was observed at all glucose concentrations studied; namely, 100, 200, 400, and 800 mg. per cent.

6. It is concluded that glucose utilization is impaired in the diabetic liver. The depression in glucose utilized for lipogenesis was far greater than the depression in CO_2 formation from the added glucose.

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INSULIN AND HEPATIC UTILIZATION OF GLUCOSE FOR LIPOGENESIS*

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In an earlier study (1) we compared surviving liver slices obtained from normal rats and from rats in which diabetes had been present for 90 to 165 days with respect to their capacity to convert C^{14} -labeled glucose to fatty acids and CO_2 . Significant amounts of the glucose- C^{14} were recovered as fatty acids when normal liver slices were incubated for 3 hours in a Ringer-bicarbonate medium. Under identical conditions the livers of the diabetic rats lost almost completely the capacity to convert added glucose to fatty acids. This failure in lipogenesis was observed at all glucose concentrations studied, namely 100, 200, 400, and 800 mg. per cent. The $C^{14}O_2$ recoveries were also depressed in the experiments with diabetic liver slices, regardless of the concentration of glucose added to the medium. In all cases, the impairment in glucose utilization for lipogenesis was far more pronounced than was that involving the conversion of the added glucose to CO_2 .

The present investigation deals with the effects of pretreatment with insulin upon utilization of glucose for lipogenesis and CO_2 in the livers of normal and diabetic rats.

Effects of Insulin Injections on Utilization of Added C^{14} -Glucose by Diabetic Liver Slices

The evidence of diabetes is presented in Table I for the rats used in Experiments 1 to 4 (Tables II and III), all twelve of which were diabetic. It should be noted that the diabetes was of long standing (63 to 200 days) and that, in these rats, *fasting* blood sugars of 173 to 374 mg. per cent were found 2 to 3 weeks before the animals were sacrificed. In the non-fasted animals that did not receive insulin, the blood sugar values at the time of sacrifice ranged from 449 to 652 mg. per cent.

In all experiments, the C^{14} -labeled glucose was added to the Ringer-bicarbonate medium in which the liver slices were incubated, and the results are expressed as percentages of this C^{14} recovered as fatty acids and

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as CO₂. The concentration of glucose in the medium was about 400 mg. per cent.

Experiment 1 (Table II)—The three diabetic rats used in this experiment were maintained on the stock diet which was high in carbohydrate. Rats D2 and D3 were injected subcutaneously with 10 units of unmodified insulin (iletin, Lilly¹) for 4 successive days and with 20 units on the morning of the 5th day. They were sacrificed about 1 hour after the last insulin injection, and their livers were sliced and incubated as described below.

TABLE I
History of Alloxan-Diabetic Rats Used in This Study

Rat		Rat weight		Duration of diabetes	Degree of diabetes				
No.	Sex	When injected with alloxan	When sacrificed		Daily food intake*	Range, urine flow in 24 hrs.†	Maximum urine sugar for 24 hr. sample‡	Fasting blood sugar††	Blood sugar at time of sacrifice‡ (not fasting)
		gm.	gm.	days	gm.	cc.	gm.	mg per cent	mg. per cent
D1	M.	250	320	100	30	70-130	8.3	193	472
D2	"	300	340	136	30	30-80	8.7	173	
D3	"	234	260	63	28	40-76	6.6	221	
D4§	F.	100	215	200	26	50-115	10.5	241	449
D5§	"	150	200	150	22	50-88	6.5	244	
D6§	"	180	200	93	18	15-48	3.0	189	544
D7§	"	140	180	65	20	50-90	5.3	374	
D8§	"	164	240	72	16	40-60	4.5	200	
D9§	M.	163	170	107	25	70-130	4.7	190	652
D10§	F.	118	166	112	28	100-190	8.9	272	
D11§	"	159	223	142	30	80-120	9.1	188	
D12§	M.	178	378	150	35	120-180	10.0	210	

* Average for amounts ingested daily during 2 weeks before sacrifice.

† Determined 1 to 3 weeks before the rats were sacrificed.

‡ Whole blood used for sugar determinations.

§ Fed Diet G for 1 or more weeks. All the others were fed the stock diet.

|| Insulin injected before the rats were sacrificed; see Tables II and III.

Rat D1 received no insulin and its liver served as the control. The results are recorded in Table II.

Utilization of the C¹⁴-glucose was greatly depressed in the liver of the control diabetic rat. Practically none of the C¹⁴ was recovered as fatty acid, and only 1.5 per cent as CO₂. The injections of insulin stimulated the formation of C¹⁴O₂ and fatty acid-C¹⁴. The values for fatty acid-C¹⁴ were 0.4 and 0.7; those for C¹⁴O₂ were 5.4 and 11.1.

¹ All the insulin used in this study was generously donated by Eli Lilly and Company.

Experiment 2 (Table II)—The two diabetic rats used in Experiment 2 had been fed a high carbohydrate diet (Diet G) for 1 week before they were sacrificed. Rat D5 was injected on 3 successive days with 10 units of unmodified insulin, and with 20 units on the 4th day. It was sacrificed 1 hour after this fourth insulin injection. It was again evident that the

TABLE II

Effect of Pretreatment with Unmodified Insulin upon Conversion of C¹⁴-Glucose to CO₂ and Fatty Acids by Livers of Diabetic Rats Fed High Carbohydrate Diets

Each incubation flask contained about 500 mg. of liver slices and 5.0 cc. of Ringer-bicarbonate buffer. Gas phase, 95 per cent O₂ and 5 per cent CO₂. Two such flasks were prepared from the liver of each rat. They were incubated at 37.5° for 3 hours. The contents of each flask were analyzed separately and the sum of the values for the two flasks are recorded below.

Experiment No.	Rat*	Pretreatment of diabetic rats				Liver slices added to 2 baths			Glucose concentration in medium†	Per cent added glucose-C ¹⁴ recovered per gm. liver as	
		Diet	Insulin injected		Blood sugar when sacrificed	Amount wet weight	Fatty acids†	Total carbohydrate†		CO ₂	Fatty acids
			days	units per day					mg. per cent		
1	D1	Stock	None	None	472	940	1.9	2.5	438	1.5	0.0
	D2	§	4	10	245	1050	6.5	4.7	438	5.4	0.37
	D3	§	4	10	168	1000	6.3	3.7	438	11.1	0.67
2	D4	G	None	None	499	1000	4.5	3.5	392	1.1	0.02
	D5	"	3	10	100	1018	4.3	3.4	392	3.5	0.50

* The weights of the rats are recorded in Table I.

† The carbohydrate content of the slices was determined on a separate aliquot of slices and therefore represents that at the start of the experiment. Fatty acid contents of the slices were determined at the end of the run.

‡ Before addition of slices.

§ Glucose was fed after insulin injections were started. The rats in Experiment 2 were not so treated because they were maintained on Diet G which contained glucose.

|| In addition to the amounts of insulin recorded above, each rat also received 20 units of unmodified insulin 1 to 2 hours before sacrifice.

insulin treatments accelerated the conversion of the *added* glucose to CO₂ and fatty acids.

Experiment 3 (Table III)—The rats were fed Diet G for 1 week before preparation of the liver slices. Two of the rats were injected subcutaneously with 8 units of *protamige zinc insulin* (Lilly) daily, for 4 or 5 successive days, and with 20 units of unmodified insulin 1 hour before sacrifice. The liver slices from Rat D6, which received no insulin, converted

about 1 per cent of the C^{14} to CO_2 and none to fatty acids. As a result of the insulin treatments, the liver slices of the other two rats converted 4.4 and 4.9 per cent of the C^{14} to fatty acids, and 9 and 12 per cent respectively to CO_2 .

Experiment 4 (Table III)—Three of the diabetic rats fed Diet G were injected daily with protamine zinc insulin for 1 to 3 days, and with 20 units

TABLE III

Effect of Pretreatment with Protamine Zinc Insulin upon Conversion of C^{14} -Glucose to CO_2 and Fatty Acids by Livers of Diabetic Rats Fed High Carbohydrate Diet

Each incubation flask contained about 500 mg. of liver slices and 5 cc. of Ringer-bicarbonate buffer. Gas phase, 95 per cent O_2 and 5 per cent CO_2 . Two such flasks were prepared from the liver of each rat. They were incubated at 37.5° for 3 hours. The contents of each flask were analyzed separately and the sum of the values for the two flasks is recorded below.

Experiment No.	Rat*	Pretreatment of diabetic rats			Liver slices added to 2 baths			Glucose concentration in medium†	Per cent added glucose, C ¹⁴ recovered per gm. liver as	
		Protamine zinc insulin injected		Blood sugar when sacrificed	Amount wet weight	Fatty acids‡	Total carbohydrate‡		CO ₂	Fatty acids
		days	units per day							
3	D6	None	None	544	1016	3.5	2.6	392	1.1	0.0
	D7	4	8½	116	1030	4.9	3.0	392	12.4	4.9
	D8	5	8½	98	995	3.3	6.1	392	9.0	4.4
4	D9	None	None	652	991	2.4	3.0	366	0.9	0.0
	D10	1	10½	299	1016	2.3	12.8	366	4.6	1.9
	D11	2	10½	173	994	3.2	7.8	366	7.3	3.6
	D12	3	10½	180	989	7.0	5.1	366	9.2	5.0

* The weights of all the rats are recorded in Table I. Diet G was fed to the rats throughout these experiments.

† See foot-note, Table II.

‡ Before addition of slices.

§ See foot-note, Table II.

of unmodified insulin 1 hour before their livers were excised. The stimulating effects of this hormone upon $C^{14}O_2$ and fatty acid- C^{14} recoveries were equally as striking as those observed in Experiment 3. It was shown in addition, however, that pretreatment of diabetic rats with insulin for only 1 day was sufficient to initiate the increased utilization of the added glucose for fatty acid and CO_2 formation.

Experiment 5—The administration of insulin for a short period of time had no effect on hepatic lipogenesis. Three diabetic rats were injected with 10 units of unmodified insulin and sacrificed 1, 2, and 3 hours later.

Their livers were sliced and incubated with C^{14} -glucose as described below. No increase in the conversion of glucose- C^{14} to CO_2 or to fatty acids was observed. Apparently, the duration of insulin action is of some importance in eliciting the hepatic response described here.

Effects of Insulin Injections on Utilization of Added C^{14} -Glucose by Normal Liver Slices

Two normal rats which had been fed the high carbohydrate Diet G for 1 week were injected subcutaneously with 8 units of protamine zinc in-

TABLE IV

Effect of Pretreatment with Protamine Zinc Insulin upon Conversion of C^{14} -Glucose to CO_2 and Fatty Acids by Liver of Normal Rats Fed High Carbohydrate Diet (Diet G)

Each incubation flask contained about 500 mg. of liver slices and 5.0 cc. of Ringer-bicarbonate buffer. Gas phase, 95 per cent O_2 and 5 per cent CO_2 . Two such flasks were prepared from the liver of each rat. They were incubated at 37.5° for 3 hours. The contents of each flask were analyzed separately and the sums of the values for the two flasks are recorded below.

Rat No.	Weight	Insulin injected into rats				Blood sugar at time of sacrifice	Liver slices added to 2 baths			Glucose concentration in medium†	Per cent added glucose-C ¹⁴ recovered per gm. liver as	
		Kind	Be-fore sacri-fice		Amount wet weight		Fatty acids*	Total car-bohy-drate*	CO ₂		Fatty acids	
			days	units per day								
	gm.			mg. per cent	mg.	per cent	per cent	mg. per cent				
21	225	None		135	1022	2.9	4.5	396	5.0	0.64		
22	200	"		115	991	3.0	5.7	400	4.5	0.81		
23	225	Protamine zinc	3	8‡	85	1014	3.6	3.9	400	12.2	6.20	
24	240	" "	10	8‡	90	1031	5.1	4.7	396	14.5	8.14	

* See foot-note, Table II.

† Before addition of slices.

‡ See foot-note, Table II.

sulin daily for 3 and 10 days, and with 20 units of unmodified insulin 1 hour before they were sacrificed (Table IV). Their blood sugar values at the time they were sacrificed were 85 to 90 mg. per cent. Two other rats served as controls; they were fed the high carbohydrate diet but were not injected with insulin. Liver slices prepared from all four rats were incubated in the presence of approximately 400 mg. per cent of glucose.

The results recorded in Table IV show that insulin injections also influenced, in quite a pronounced manner, the utilization of the added C^{14} -glucose by the normal liver. The recoveries of fatty acid- C^{14} were in-

creased 10-fold. The recoveries of $C^{14}O_2$ were somewhat more than doubled under the influence of the hormone.

DISCUSSION

Stetten and his associates were the first to show, by means of deuterium, depressed lipogenesis in alloxan-diabetic rats (2) and rabbits (3). They also reported that the administration of insulin to a normal rabbit fed a high carbohydrate diet increased hepatic lipogenesis (3). This rabbit had received two insulin injections during the 48 hours before it was sacrificed. They were unsuccessful, however, in demonstrating increased hepatic lipogenesis in normal rats 3 hours after the injection of insulin (4). As noted above, the duration of insulin action is of importance in eliciting a response of the liver slice to injected insulin. The short interval that elapsed between the time of insulin injection and the time of sacrifice may explain the failure of Stetten and Klein to find increased lipogenesis in their rats.

Two factors have now been shown to control the rate of lipogenesis from carbohydrate in the liver. These are previous carbohydrate intake (5) and insulin. The conversion of glucose to fatty acids proceeds readily in the liver of the *normal* rat provided it is fed a diet containing adequate amounts of carbohydrate. When rats are fasted or fed diets low in carbohydrate, their livers lose, in large measure, the ability to effect this conversion. Even the liver of the diabetic rat fed a high carbohydrate diet does not convert glucose to fatty acids (1). Insulin not only corrects this defect in glucose utilization in the diabetic rat, but also stimulates lipogenesis in the liver of the normal rat fed a high carbohydrate diet.

It is in the studies with the normal liver slices that the full extent to which lipogenesis can serve as a pathway in glucose utilization is revealed. As much as 8 per cent of the added glucose- C^{14} was recovered as fatty acids in the experiments with livers of normal rats that had been pretreated with insulin, and this quantity of C^{14} is about half the amount that was recovered as CO_2 .

The mechanisms by which insulin influences glucose utilization in the liver are not well understood. We have found that pretreatment with insulin increases the conversion of the labeled glucose to glycogen as well as to fatty acids and CO_2 in the liver. These findings are consistent with the view that insulin acts upon hepatic utilization of carbohydrate at the level of the hexokinase reaction, as postulated by Cori and Cori (6). Another action of insulin, directly on the conversion of a glucose intermediate to fatty acids, is not ruled out, particularly in view of the observation that hepatic utilization of glucose for lipogenesis is stimulated to a greater degree than is CO_2 formation.

EXPERIMENTAL

Rats of the Long-Evans strain were used throughout. They were fed either the stock diet or Diet G. Both diets were high in carbohydrate, but only the latter contained free glucose.²

Induction of Diabetes—Rats were made diabetic by an intravenous injection of a 5 per cent solution of alloxan monohydrate (50 mg. per kilo, body weight). Their subsequent treatment has already been described in detail (1). The diabetic rats used in this study exhibited polyuria and glycosuria and, with few exceptions, had fasting blood sugar levels in excess of 200 mg. per cent. The histories of the diabetic rats are shown in Table I.

Radioactive Glucose—Radioactive glucose was prepared photosynthetically by the method of Putman *et al.* (9). We are indebted to Mr. S. Abraham for its preparation. The sample of labeled glucose used in this study was shown to be uniformly labeled.³

Incubation Procedures—The incubation procedures have been described elsewhere (1, 10). The rats were sacrificed by a blow on the head. Liver slices were incubated in an oxygenated Ringer-bicarbonate buffer containing about 400 mg. per cent of glucose. The incubation flask was designed to permit the collection of CO₂ at the end of the experiment (10).

Analytical Procedures—The methods of analyses used in this study were essentially the same as those described in a previous report (1). However, the C¹⁴ content of the lipide fraction was determined without previous removal of the unsaponifiable material. Repeated tests have shown that less than 5 per cent of the C¹⁴ in the lipide fraction is precipitable with digitonine.

SUMMARY

1. The effects of previous insulin injections, in normal and alloxan-diabetic rats, upon the capacity of their livers to convert added glucose to fatty acids and CO₂ were studied. Slices were prepared from these livers and incubated in a Ringer-bicarbonate medium containing 400 mg. per cent of glucose labeled with C¹⁴-glucose, and the recoveries of the C¹⁴ in fatty acids and CO₂ were measured at the end of 3 hours. The diabetes

² Composition of stock diet, 68.5 per cent whole wheat; 5 per cent casein; 10 per cent fish-meal; 1.5 per cent salt; 5 per cent fish oil; and 10 per cent alfalfa. This diet contained approximately 50 per cent carbohydrate and 22 per cent protein. Composition of Diet G, 60 per cent glucose; 22 per cent casein (Labco, vitamin-free); 6 per cent salt mixture (7); 12 per cent Cellu flour. Vitamins A and D were added as a fish oil. The other vitamins (8) were mixed with the Cellu flour.

³ Personal communication from Dr. W. Z. Hassid.

of the rats used in this study was of long standing (63 to 200 days), and their fasting blood sugars, determined 2 to 3 weeks before they were sacrificed, were 173 to 374 mg. per cent.

2. The livers of the alloxan-diabetic rats converted little or none of the added C^{14} -glucose to fatty acids. Pretreatment of such rats with insulin for 1 to 5 days completely repaired this defect in utilization of carbohydrate for lipogenesis. This action of the hormone was demonstrated even in a rat in which diabetes had existed for as long as 150 days.

3. Pretreatment of normal rats with insulin for 3 and 10 days resulted in a 10-fold increase in the capacity of the liver to convert added glucose to fatty acids.

4. Insulin injections also caused a pronounced increase in the capacity of the normal and diabetic livers to oxidize added C^{14} -glucose to CO_2 . But in no case was this effect of the hormone so striking as in its effect upon lipogenesis.

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CHOLESTEROL ESTERASES

IV. CHOLESTEROL ESTERASE OF RAT INTESTINAL MUCOSA*

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Nieft and Deuel (1, 2) found that in rat liver and intestinal mucosa two different opposing systems were responsible for the synthesis and hydrolysis of cholesterol esters. The esterifying system required the presence of phosphate ion and a fatty acid source. The hydrolytic system consisted of at least two factors and its activity was accentuated by soy bean lecithin. While their extracts were prepared in a standard manner and were of constant concentration, they report that it was impossible to tell beforehand which would be the predominant reaction in any given extract. Esterification by extracts of intestinal mucosa could be demonstrated only when the animals had been fed a diet containing 1 per cent lanolin or 1 per cent cholesterol for 2 or 3 weeks previous to sacrifice. Extracts of the intestines of such animals initially exhibited only esterifying activity. However, dialysis against distilled water for 24 hours inactivated the esterifying system and then the hydrolytic system could be demonstrated.

Recent papers (3-5) from this laboratory have reported procedures for studying the esterifying and hydrolyzing cholesterol esterase systems and the occurrence and characteristics of both systems in pancreatin and dog serum. The properties of the enzyme in pancreatin appeared to be favorable for activity in the lumen of the small intestine; namely, optimum pH in the range of 6 to 7 and activity in the presence of bile salts.¹ In view of the papers by Mueller (6), Frölicher and Süllmann (7), and Schramm and Wolff (8), who have suggested that either hydrolysis or esterification or both are important in the absorption of cholesterol, it seemed desirable to investigate further the occurrence of cholesterol esterase in the intestinal mucosa. Accordingly, extracts of rat intestine were

* This work was performed under Contract No. 74400 between George Washington University and the Office of Naval Research, United States Navy Department.

¹ It should be pointed out that in the present report and in the earlier ones from this laboratory (3-5) cholesterol oleate and cholesterol and oleic acid have been used as the substrates for hydrolysis and esterification respectively. Unpublished experiments suggest that either the optimum conditions for activity vary with the fatty acid component or that there are two or more different cholesterol esterase systems.

prepared under conditions which excluded contamination with pancreatic tissue or intestinal contents. Such extracts exhibited esterifying and hydrolyzing activity. The enzyme had the same characteristics as the one previously demonstrated in pancreatin (3, 4). These findings posed the interesting possibility that the enzyme in the intestinal mucosa originated in the pancreas and, after secretion into the lumen of the intestine, entered the cells of the mucosa or was so firmly adsorbed to these cells that our washing technique did not remove it. This possibility and the influence of diet on the level of cholesterol esterase activity in the mucosa were then investigated. Normal rats and rats 95 per cent depancreatized were placed on either a standard laboratory chow, a synthetic diet containing 27 per cent fat, or the synthetic diet plus 1 per cent cholesterol for 3 weeks. Assay of intestinal extracts prepared from these rats indicated the following: first, that the enzyme was markedly decreased in the 95 per cent depancreatized rats, secondly, that the cholesterol content of the diet did not influence the level of activity, and, thirdly, that increasing the fat content of the diet produced a higher level of activity in the mucosa.

EXPERIMENTAL

Animals and Diets—All the rats used were adults from our stock colony. The data on normal rats shown in Tables I, II, and III were obtained with non-fasted animals fed a commercial laboratory chow (approximately 5 per cent fat). In the experiment on the effects of diet and 95 per cent pancreatectomy the animals were housed in individual cages and received the diets and water *ad libitum*. Diet A was composed of 20 per cent casein, 5 per cent salt mixture (9), 2 per cent Cellu flour, 23 per cent starch, 23 per cent sucrose, 25 per cent lard, and 2 per cent cod liver oil. Diet B was the same as Diet A except that 1 per cent cholesterol was introduced at the expense of carbohydrate. Adequate amounts of crystalline vitamins were added to both diets. The 95 per cent pancreatectomized rats were prepared as described by Foglia (10) and placed on the experimental diets 5 to 7 days after operation.

Preparation and Assay of Extracts of Intestinal Mucosa—The rats were sacrificed by decapitation. The proximal end of the small intestine was severed just posterior to the attachment of the mesentery containing the gastroduodenal portion of the pancreas. The small intestine was then stripped of its mesentery to within 2 cm. of the ileocecal valve and removed. The duodenal end was attached to a 50 cc. burette and the opposite end was clamped off. The burette and intestine were then filled with saline and the clamp was released to allow a slow flow of saline through the intestine. The relatively constant head of pressure distended the

intestine and insured complete exposure of the surface of the mucosa. After approximately 200 cc. had been used, the position of the segment was reversed and an additional 150 cc. were run through. The intestine was dried between blotting paper and exactly 5 gm. were taken, starting from the duodenal end. The tissue was transferred to a homogenizing tube (11), chilled to 2°, and, after addition of 15 cc. of 50 per cent glycerol, phosphate buffer, or saline, homogenized for 2 minutes. The homogenate was diluted to 25 cc., shaken for 1 hour at room temperature, and then centrifuged for 5 minutes at 2500 r.p.m. The supernatant was used as the enzyme extract. 2 cc. of the extract, equivalent to 0.4 gm. of fresh tissue, were used in the assays for activity, according to our previous procedures (3, 4). In all of the experiments the substrate mixture (11 cc.)

TABLE I
Cholesterol Esterase Activity of Extracts of Rat Intestinal Mucosa

Enzyme extract No.	Preparation and treatment of enzyme extract	Enzymatic activity*	
		Esterification	Hydrolysis
		mg.	mg.
2	Glycerol-water (1:1)	25.3	13.0
2	“ heated†	0.0	0.0
3	0.154 M phosphate buffer	15.0	4.2
4	Saline	28.0	9.0
4	“ aged 24 hrs.	26.5	9.0
4	“ dialyzed 24 hrs. against saline	23.0	6.0

* Expressed as mg. of cholesterol esterified or liberated by hydrolysis per gm. of tissue per 24 hours.

† 15 minutes at 65°.

for hydrolysis contained 42 mg. of cholesterol oleate and, for esterification, 25 mg. of cholesterol and 54.6 mg. of oleic acid. The incubation time was 24 hours.

Results

Cholesterol Esterase Activity in Intestinal Mucosa of Normal Adult Rats—Preliminary tests indicated considerable hydrolyzing and esterifying activity in the mucosa. Extracts from twenty normal rats were tested and both types of activity were present in every extract. Representative experiments are shown in Tables I, II, and III. Heating the extracts for 15 minutes at 65° inactivated both esterifying and hydrolyzing systems (Table I). The loss in activity of saline extracts during dialysis against saline for 24 hours at 5° was of small magnitude and likely due to manipu-

lative loss rather than removal of a cofactor. The data in Table II show that both systems required bile salt for activity and that they were equally active in the presence of phosphate or citrate buffer. The optimum pH for esterification was 6.2 and for hydrolysis 6.5 (Table III). These are essentially the same results as those found for the pancreatic enzyme (3, 4). It will be noted that esterification was zero at pH 6.8; this was also observed with the enzyme in pancreatin. Our data on the synthesis and hydrolysis of cholesterol esters by extracts of rat intestinal mucosa

TABLE II

Effect of Changes in Substrate Mixture on Activity of Cholesterol Esterase in Extracts of Intestinal Mucosa

Enzyme extract No.	Substrate mixture		Enzymatic activity*	
	Sodium taurocholate	Buffer	Esterification	Hydrolysis
			mg.	mg.
1	+	Phosphate	17.8	6.2
1	-	"	0.0	0.0
12	+	"	25.3	13.0
12	-	"	0.0	0.0
12	+	Citrate	23.8	12.3

* See Table I.

TABLE III

Influence of pH on Activity of Cholesterol Esterase of Rat Intestinal Mucosa*

pH of digesta	5.5	5.8	6.2	6.3	6.5	6.8
	mg.	mg.	mg.	mg.	mg.	mg.
Esterification ..	14.0	14.5	18.3	16.5	9.5	0.0
Hydrolysis	4.0	5.3	6.0	8.0	9.0	8.2

* See Table I.

are not in agreement with several of the observations by Niefert and Deuel (1, 2). It does not seem likely that the use of different strains of rats could account for these differences. Conversion of the highest values for hydrolysis and esterification reported by Niefert and Deuel into mg. of cholesterol per gm. of tissue shows a maximum of 0.76 mg. esterified and 0.36 mg. hydrolyzed, or only about 1/30 and 1/40 respectively of the activity observed in our experiments with saline extracts (Table I). It is possible that the enzyme demonstrated in our experiments was not present in their extracts, which were prepared by grinding the intestine with sand under an equal volume of saline, followed by centrifuging lightly, whereas

our extracts were obtained by homogenizing, shaking for 1 hour, and then centrifuging. At the present time it is suggested that either the conditions employed by Niefert and Deuel were not optimum for the enzyme or that distinctly different systems of low activity were responsible for their results.

Influence of Diet and 95 Per Cent Pancreatectomy on Cholesterol Esterase Content of Rat Intestinal Mucosa (Table IV)—No difference was found between normal or 95 per cent depancreatized rats receiving Diet A and comparable animals on the 1 per cent cholesterol diet (Diet B). Thus, the data are not in agreement with the report (1) that esterifying activity was present in the intestine only when cholesterol was fed. The data do suggest that the fat content of the diet influences the level of both esterify-

TABLE IV

Influence of Diet and 95 Per Cent Pancreatectomy on Cholesterol Esterase Activity of Rat Intestinal Mucosa

[No. of rats]	Type	Diet	Enzymatic activity*	
			Esterification	Hydrolysis
			mg.	mg.
4	Normal	Stock	13.9 (10.5-18.8)†	11.9 (7.3-17.5)
4	Depancreatized	"	5.7 (2.5-11.8)	4.8 (2.0- 8.5)
5	Normal	A	22.5 (15.5-31.5)	15.4 (13.0-19.0)
4	"	B	29.4 (15.8-37.8)	18.6 (10.5-24.8)
4	Depancreatized	A	7.7 (4.0- 9.3)	7.3 (6.3- 8.8)
4	"	B	6.6 (3.0-10.0)	6.8 (4.5- 9.5)

* See Table I.

† The figures in parentheses indicate the range of the values.

ing and hydrolyzing activity, as may be seen by comparing the normal animals on the chow diet (approximately 5 per cent fat) with those fed the synthetic diets.

In evaluating the effect of 95 per cent pancreatectomy it should be emphasized that the animals were maintained for approximately 28 days after operation; thus, there was ample time for maximum compensation by the pancreatic remnant. Therefore, it is concluded that the pancreas is the major or sole source of this cholesterol esterase of rat intestinal mucosa. If this is so, then in the completely pancreatectomized rat there should be neither synthesis nor hydrolysis of cholesterol esters during digestion and absorption. It may be of significance in this connection that Chaikoff and Kaplan (12) have observed that in depancreatized dogs cholesterol esters may be absent in the blood. We have not been able to study the completely pancreatectomized rat owing to the difficulties of preparing and maintaining such animals.

SUMMARY

Hydrolyzing and esterifying cholesterol esterase systems have been demonstrated in the intestinal mucosa of normal adult rats. The optimum pH for hydrolysis was 6.5 and for esterification, 6.2. The enzyme required bile salts for activity and was inactivated by heating for 15 minutes at 65°. The activities were the same in phosphate or citrate buffer. The level of activity was increased on a high fat diet but incorporation of 1 per cent cholesterol in the diet did not influence the activity. Data obtained on rats depancreatized 95 per cent suggested that the pancreas was the major or sole source of the enzyme in the mucosa.

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THE DETERMINATION OF COLLAGEN AND ELASTIN IN TISSUES

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In the study of the occurrence and distribution of collagen in normal and pathological tissue and its formation and development in the embryo there has been need for an accurate method to determine this substance in small amounts of tissue. Spencer, Morgulis, and Wilder (1) devised a method based on conversion of collagen to gelatin followed by the determination of the nitrogen of the gelatin precipitated with tannic acid. There is no assurance that only gelatin is extracted and precipitated under the given conditions. Later work (2) has indicated that excess nitrogenous material is measured by this method.

Lowry, Gilligan, and Katersky (2) developed a gravimetric procedure involving extraction of soluble non-collagenous substances with 0.1 N NaOH at room temperature and conversion of collagen to gelatin by autoclaving; collagen was determined by difference in the dry weight of the residues. It was necessary to assume that different tissues behaved alike under the standard conditions and that the various fractionating procedures specifically effected the separation of the different components of the tissues. Abercrombie and Johnson (3) obtained an extract of nerve tissue by autoclaving according to the procedure of Lowry *et al.* (2) and calculated collagen from the nitrogen content of the extract. Elastin has been determined by weighing the fraction of tissue resistant to solution in 0.1 N NaOH at 100° (2).

A simple method is described here for determining collagen in small amounts of tissue with a precision and accuracy usually better than ± 5 per cent. In tissues containing but little collagen much of the soluble protein is separated from the tissues with the aid of 20 per cent urea solution. (Tissues containing large amounts of collagen do not require this preliminary separation.) The collagen is converted to soluble form (gelatin) by hydrolysis with water in an autoclave and thus extracted from elastin and other substances not dissolved by the process. The collagen content is estimated from the hydroxyproline content of acid hydrolysates of the extract.

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As previously shown (4), the hydroxyproline content of collagen from various mammalian or avian sources is 13.5 ± 0.24 per cent and apparently is present in significant amounts in no other protein except elastin.

Elastin, which contains 1.5 to 2.3 per cent hydroxyproline, depending on the source, is estimated from the hydroxyproline content of the residue remaining after extraction of the collagen.

Procedure

The collagen content of tissues is expressed as per cent of dry fat-free weight. To 100 to 1000 mg. of finely minced tissue are added 15 ml. of acetone. After 6 hours or more the acetone is decanted and replaced by 15 ml. of fresh acetone which is allowed to stand 6 hours or more. This is followed by extraction with 15 ml. of ether for 12 to 16 hours. The residue is dried to constant weight at 108° .

For the extraction of collagen, 5 to 100 mg. portions of dry fat-free tissue high in collagen (skin, aorta, chordae tendineae, bone) are accurately weighed and placed in 15 ml. centrifuge tubes with 4 ml. of water. The tubes are stoppered with cotton and gauze plugs and autoclaved 3 hours at 15 pounds pressure. The solution is centrifuged until clear if necessary and transferred to 18×150 mm. test-tubes. The residue is washed with 4 ml. of water which, after centrifuging, is transferred to the test-tube. The residue in the centrifuge tube is again autoclaved with about 4 ml. of water for 3 hours. In the meantime, the supernatant in the test-tube is evaporated almost to dryness by directing a stream of air into the tube placed in a boiling water bath. The supernatant from the second autoclaving and two washings with hot water are transferred to the test-tube and then evaporated to dryness.

To prepare hydrolysates of the extracted collagen, 1.0 ml. of 6 N HCl is added for each estimated 50 mg. of extracted protein. The tubes are sealed and autoclaved 3 hours at 50 pounds pressure. The hydrolysates are neutralized with NaOH solution, diluted to appropriate volume (35 to 120 γ of hydrolyzed collagen per ml.), and filtered. Hydroxyproline is determined according to the procedure previously described (4).

Tissues low in collagen (muscle, kidney, spleen) require a preliminary extraction of non-collagenous proteins. This preliminary extraction is accomplished by grinding 100 to 1500 mg. of the fresh tissue (dry fat-free weight being determined from other samples of the tissue) with sand and 20 per cent urea solution in a mortar. The mixture is suspended in about 40 to 45 ml. of urea solution at room temperature in a 50 ml. centrifuge tube for 1 hour with occasional stirring. The tubes are centrifuged and the supernatant is discarded. The residue is washed three times with 45 ml. of water, and transferred to a 15 ml. centrifuge tube with the aid

of about 10 ml. of water which is removed by centrifugation and discarded. The residue is stirred with 4 to 5 ml. of water and autoclaved. The subsequent procedure is as described above.

The hydroxyproline content of collagen preparations from different mammalian and avian sources has been found to be 13.4 ± 0.24 per cent (4). Hydroxyproline may be converted to its equivalent of collagen through multiplication by the factor 7.46. The percentage of collagen of a tissue sample may be expressed by the relation

$$\frac{\text{Micrograms hydroxyproline in 1 ml. hydrolysate}}{\text{Micrograms tissue represented in 1 ml. hydrolysate}} \times 7.46 \times 100$$

As a measure of protein other than collagen remaining in extracts used for estimation of collagen, the tyrosine content of hydrolysates of the extracts was determined with the aid of the Folin-Ciocalteu phenol reagent according to the method of Heidelberger and MacPherson (5). The Millon-Lugg procedure as modified by Block and Bolling (6) yielded essentially similar values. The hydroxyproline values of extracts and residues for collagen and elastin estimations were corrected for the color contributed by tyrosine.

To determine elastin, the residues remaining from the collagen determination (which had been autoclaved for two 3 hour periods and washed after each autoclaving) are autoclaved for a third 3 hour period and again washed with 8 ml. of water. The residue is then hydrolyzed for 3 hours at 50 pounds pressure with 6 N HCl, and the hydroxyproline determined. The values are corrected for tyrosine present.

The per cent of elastin is calculated from the expression (micrograms of hydroxyproline)/(micrograms of sample) \times factor \times 100. The factor is 66.7 for pig elastin (1.50 per cent hydroxyproline), 52.3 for beef elastin (1.91 per cent hydroxyproline), and 43.4 for rat elastin (2.30 per cent hydroxyproline).

Collagen in tissues is contained in tendons, membranes, or in fibrils forming a network within structures. The content in a muscle may be expected to increase as the locations of the samples taken for analysis approach the fibrous attachments.

Comparison of the collagen content of different tissues necessitates the analysis of representative samples of entire organs or careful definition of the location of the sample taken to represent a constituent part.

Most of the analyses reported in this paper were carried out to test the reproducibility of the procedure on small contiguous samples of similar gross appearance from muscles, or organs of large animals, or minced portions of the entire organs of small animals.

From the muscles of large animals, areas near the attachments were

avoided and membranous surfaces discarded to obtain uniform samples of tissue. Similarly, portions of apparently uniform beef or pig kidney cortex or liver tissue were dissected. On the other hand, the entire ventricles, liver lobes, and spleen of the rat were used for analysis.

Results

The collagen content of samples of tissue determined by the present procedure is recorded in Table I. The values ranged from 85 per cent collagen content for beef chordae tendineae to 0.22 per cent for rat brain. The average deviation from the mean of the determinations was usually less than ± 5 per cent of the mean. The correction for tyrosine in the majority of samples was less than 1 per cent and never greater than 3 per cent of the collagen value.

Also in Table I is shown the elastin content of samples of tissues as determined by the present procedure. The values ranged from 57 per cent for pig aorta to 0.5 per cent for rat kidney cortex. Liver, brain, and myocardium did not contain measurable amounts of elastin. The average deviation from the mean of the values was usually less than ± 5 per cent of the mean. Tyrosine corrections were 2 to 20 per cent of the elastin values.

DISCUSSION

Autoclaving several different tissues (except bone and teeth) for 2 to 4 hours resulted in the extraction of 93 to 98 per cent of the collagen. Autoclaving for two 3 hour periods removed 99 per cent or more of the collagen, and the extracts from an additional 3 hours contained less than 1 per cent as indicated by hydroxyproline determinations. Tissues were autoclaved for two 3 hour periods (rather than one 6 hour period) and the supernatant liquid decanted after each autoclaving to insure complete removal of any gelatin which might be held in the insoluble residue.

Undecalcified bone and teeth retained 5 to 9 per cent of their hydroxyproline after 6 hours and 3 per cent after 12 hours of autoclaving. The total hydroxyproline extracted, as well as that retained in the residues, could be obtained from the determinations made on hydrochloric acid hydrolysates of the whole tissue.

Autoclaving of whole tissues removes proteins which contain tyrosine along with the collagen. Because tyrosine produces 1.5 per cent as much color as does hydroxyproline in the determination, it is advisable to remove much of the extraneous protein from tissues which contain but little collagen by a preliminary extraction with 20 per cent urea solution.

Tissues high in collagen or containing but small amounts of other pro-

TABLE I
Collagen and Elastin Content of Tissues

Tissue	Collagen* (uncorrected)	Tyrosine correction	Collagen* (corrected)	Average deviation from mean	Elastin* content	Average deviation from mean
	per cent	per cent of total color	per cent	per cent	per cent	per cent
Beef aorta (arch).....	23.2 (4)†	0.60	23.1	2.1	39.8 (2)†	1.1
“ “ “ †.....					37.8 (2)	0.50
Pig “ “	16.1 (4)	0.65	16.0	3.1	57.1 (1)	
“ “ “ †.....					53.4 (4)	4.2
Rat “ “	25.7 (2)	0.40	25.6	2.3	47.7 (2)	0.33
“ “ “ †.....					47.0 (2)	1.38
Beef bone (tibia)§.....	24.2 (4)	0.11	24.2	0.43		
Rat “ (femur)§.....	15.1 (4)	0.15	15.1	2.5		
Beef chordae tendineae (large).....	84.8 (5)	0.25	84.6	0.78	4.88 (1)	
Pig chordae tendineae (large).....	77.1 (2)	0.27	76.9	3.5	3.69 (1)	
Beef liver 	1.99 (4)	1.12	1.97	9.0	0 (2)	
Pig “ 	2.49 (5)	1.21	2.46	5.1	0 (2)	
Rat “ 	0.65 (3)	2.15	0.64	5.1	0 (2)	
Beef kidney cortex 	5.30 (4)	0.39	5.28	2.8	1.65 (2)	2.7
Pig “ “ 	3.82 (5)	0.43	3.80	3.9	0.58 (2)	2.8
Rat “ “ 	3.35 (3)	0.57	3.33	0.99	0.45 (2)	6.0
Beef muscle (shoulder)	2.14 (4)	2.90	2.08	2.3		
Rat “ (abdominal)	5.83 (3)	0.98	5.77	4.1		
Beef myocardium (left ven- tricle) 	1.99 (5)	2.96	1.93	2.8	0 (2)	
Pig myocardium (left ven- tricle) 	2.24 (5)	2.86	2.18	2.2	0 (2)	
Beef myocardium (right ven- tricle) 	3.82 (4)	1.56	3.76	3.4	0 (2)	
Pig myocardium (right ven- tricle) 	3.45 (2)	2.00	3.38	0.90	0 (2)	
Rat ventricle 	3.00 (3)	1.44	2.96	2.7	0 (2)	
Beef spleen 	3.12 (4)	0.75	3.10	2.9	4.55 (2)	5.1
Pig “ 	2.42 (4)	0.82	2.40	6.1	1.25 (2)	8.0
Rat “ 	3.52 (2)	0.57	3.50	0.85	0.55 (2)	15.0
Rat brain (cerebrum) 	0.22 (2)	1.80	0.22	2.3	0 (2)	
“ duodenum 	12.0 (2)	0.38	12.0	0.0		
“ lung 	11.3 (2)	0.44	11.3	1.7	4.89 (2)	1.04
“ stomach (cardia) 	23.7 (3)	0.40	23.6	4.6	1.64 (3)	12.9
“ “ (pylorus) 	13.9 (3)	0.52	13.8	6.5	1.27 (2)	2.8
“ teeth (incisor)§.....	10.8 (2)	0.16	10.8	1.4		
“ skin (side).....	67.8 (4)	0.26	67.6	1.54		

TABLE I—*Concluded*

Tissue	Collagen* (uncorrected)	Tyrosine correction	Collagen* (corrected)	Average deviation from mean	Elastin* content	Average deviation from mean
	<i>per cent</i>	<i>per cent of total color</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Dog skin†... ..	64.5 (2)	0.34	64.3	0.46		
Guinea pig skin‡... ..	72.3 (2)	0.21	72.1	0.41		
Human skin**... ..	72.1 (3)	0.25	71.9	0.69		

* Based on dry fat-free weight.

† The figures in parentheses indicate the number of samples.

‡ Based on weight of residues from autoclaving and NaOH treatment.

§ Autoclaved for 12 hours.

|| Extracted with 20 per cent urea before autoclaving.

¶ Kindly supplied by Dr. Wm. A. Altemeier, Department of Surgery, University of Cincinnati College of Medicine.

** Kindly supplied by Dr. R. R. Suskind, Department of Dermatology, University of Cincinnati College of Medicine.

tein made soluble by autoclaving (skin, bone, aorta, chordae tendineae) do not require preliminary extraction.

Tyrosine-containing proteins were present in extracts made by autoclaving whole tissues of low collagen content (muscle, liver, spleen, brain, kidney) in amounts sufficient to produce color equivalent to 2 to 9 per cent of that from the hydroxyproline. After preliminary extraction with 20 per cent urea solution, the tyrosine remaining usually accounted for much less than 2 per cent of the total color formation, except in the case of muscle tissue, especially heart muscle, in which the remaining tyrosine color amounted to almost 3 per cent of the total (Table I).

The hydroxyproline content of the residues obtained after urea extraction were found to be equal to that of the whole tissues tested (Table II).

Preliminary extraction of heart muscle tissue overnight with 0.1 N sodium hydroxide did not remove appreciably greater amounts of the extraneous tyrosine-containing proteins than did urea. This extraction furthermore introduced the necessity of a tedious extraction of the NaOH from the residue which had been made viscous by the swelling of the nucleates. Occasionally, low values for collagen were obtained either as a consequence of mechanical losses or through the dissolution of part of the collagen by the alkali.

It is not presumed that the precision of the elastin determination by this or any other procedure available at present is equal to that of the collagen analyses. There is good evidence that elastin is measured by it

with adequate reproducibility to indicate changes which may occur in tissues under normal and pathological conditions.

The hydroxyproline content of samples of elastin prepared by different procedures, although small, has been found to be essentially similar within the limits of the analytical method. For instance, the hydroxyproline contents of pig and beef aorta were respectively 1.50 to 1.91 per cent, whether the tissues were autoclaved for three 3 hour periods and washed after each autoclaving and then heated at 100° for 30 minutes with 0.1 N NaOH or heated at 100° for 40 hours with urea solution.

Previous procedures for the determination of elastin rested on the assumption that the residue after extraction with 0.1 N NaOH contained all

TABLE II
Comparison of Collagen Content Found in Tissues with and without Preliminary Extraction with Urea Solution

Tissue	Tyrosine correction, per cent total color		Collagen found,* per cent	
	Sample A†	Sample B‡	Sample A†	Sample B‡
Beef spleen.....	5.7	1.0	3.34	3.29
Pig "	6.9	0.1	3.25	3.17
Beef kidney cortex.....	7.0	0.7	6.82	7.03
Pig "	7.0	0.7	3.27	3.44
Beef myocardium, left ventricle..	6.9	2.8	2.16	2.19

* Corrected for tyrosine color.

† Sample A, whole tissue.

‡ Sample B, extracted with 20 per cent urea solution.

the original elastin and no other substances, because the elastin was estimated from the weight of the residue.

Treatment of myocardium (residues) with 0.1 N NaOH at 100° for 30 minutes left a weighable residue which, however, contained no hydroxyproline and therefore was not elastin. Similar treatment of kidney residues, in which the elastin present is known to be in fine fibrils, produced results which were considerably lower than those obtained from the hydroxyproline content of thrice autoclaved samples. It is reasonable to presume that the lower values determined after NaOH treatment may have been due to partial solution of the fine fibrils by the NaOH.

In the procedure described here, it is only necessary to assume that collagen is separated quantitatively from the elastin. Extraneous proteins which contain no hydroxyproline do not influence the results.

The amount of hydroxyproline contained in the third extraction after autoclaving would increase the determined elastin value of aorta 1 per

cent, and as much as 20 per cent in kidney in which the elastin content is small (0.6 per cent) and the collagen content relatively high (4 per cent). The amount removed in each extraction (90 per cent of the amount in the residue) corresponds to that expected on a clear separation of an insoluble moiety from a soluble portion. Consequently the error due to retained collagen in the precipitate can be estimated to be not greater than 2 per cent. After the third autoclaving of liver, brain (cerebrum), and myocardium, a residue remained which was comparable in amount to that obtained from other tissues. These residues contained no measurable amount of hydroxyproline and therefore insignificant amounts of elastin, results which are in keeping with the histological examination of these tissues.

SUMMARY

1. A colorimetric method of determining the collagen content of tissues is described.
2. Means are provided for estimating the effectiveness of separation of collagen from tissue by autoclaving.
3. A method of estimating the elastin content of tissue is described.
4. The collagen content of different tissues of the cow, pig, and rat, and of human, guinea pig, dog, and rat skin as determined by the present method is reported.
5. The estimated elastin content of different tissues is reported.

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ON THE STRUCTURE OF TRIPHOSPHOPYRIDINE NUCLEOTIDE

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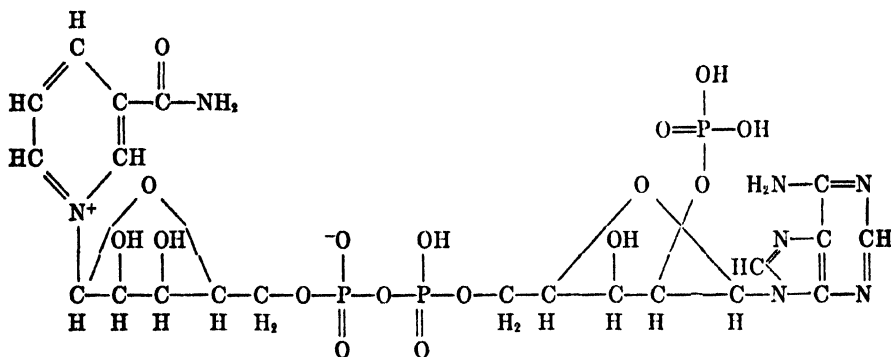
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As a result of the work of Warburg (1), von Euler (2), and their associates, it is known that triphosphopyridine nucleotide (TPN) and diphosphopyridine nucleotide (DPN) are dinucleotides and differ only in that TPN contains one additional phosphate group. The structure proposed by Schlenk and von Euler for DPN, in which nicotinamide ribose phosphate and adenosine-5-phosphate are linked by a pyrophosphate bond, has been confirmed by subsequent studies (3-5). The findings of von Euler and coworkers (6) of an interconversion of TPN and DPN in yeast maceration juice provided further evidence that these nucleotides were very similar in structure and it remained only to determine the disposition of the additional (third) phosphate group of TPN. It was considered that the three phosphates were either linked in a chain (7) or, as later suggested by Schlenk *et al.* (8), that the third phosphate group was esterified to the pentose of the adenosine portion of the molecule. The unavailability of adequate amounts of pure TPN postponed a definitive solution of this question (9). Recently, a study of the action of nucleotide pyrophosphatase (10, 4), an enzyme which breaks the pyrophosphate bonds in several nucleotides, revealed that the products of TPN cleavage were nicotinamide ribose phosphate and a diphosphoadenosine fragment which was not adenosine pyrophosphate.

The present paper is a study of the structure of this diphosphoadenosine fragment. On the basis of evidence already available it appears rather certain that one of the phosphates of the diphosphoadenosine fragment is esterified at carbon 5 of the ribose. DPN has been shown to contain adenosine-5-phosphate and there is accumulating evidence for a direct interconversion of DPN and TPN. In addition to the studies with yeast juice (6), potato extracts are known to convert TPN to DPN (4) and the mechanism of synthesis of TPN from DPN has been shown with a purified yeast enzyme to involve a direct phosphorylation by ATP (11). Also, TPN cleavage by washed kidney particles (12) yielded adenosine-5-phosphate in almost stoichiometric amounts (unpublished experiments). Concerning the point of attachment of the remaining phosphate, carbons 2 and 3 are the only likely sites, since carbon 1 is in glycosidic linkage with adenine and carbon 4 is part of a furanose ring. As a result of fur-

ther investigation it has been possible to hydrolyze specifically the phosphate esterified to carbon 5 of the diphosphoadenosine fragment and thus permit a comparison of the properties of the remaining adenosine monophosphate with adenosine-2-phosphate and adenosine-3-phosphate. The identification was greatly facilitated by the important discovery by Carter and Cohn (13) that adenylic acid derived from yeast nucleic acid is actually a mixture of nearly equal parts of adenosine-3-phosphate and a second adenylic acid (*a*) which in all likelihood is adenosine-2-phosphate. We have found that the adenylic acid derived from TPN degradation is different from adenosine-3-phosphate but is indistinguishable from adenylic acid *a*. Thus it appears that the accompanying structure is the most probable for TPN.



Methods

TPN prepared by the method of LePage and Mueller (14) resulted in final products which varied in purity from 73 to 78 per cent. The analysis, stated in moles per mole of TPN (assayed enzymatically (4)), was as follows: total phosphate 3.02, orthophosphate 0.00, light absorption at 260 $m\mu$ (with use of a molar extinction coefficient of 20,000) 0.98, nicotinamide-ribose moiety (15) 1.17, and pentose 2.53. Since the phosphate-TPN molar ratio established the absence of phosphate-containing impurities in this TPN sample, it was considered suitable for use in the present investigation.

Crystalline adenosine-5-phosphoric acid was obtained from the Sigma Chemical Company. Recrystallized adenylic acid *a* and adenosine-3-phosphoric acid were prepared in this laboratory by the ion exchange technique of Cohn (16) or were generously furnished by Dr. C. E. Carter and Dr. W. E. Cohn.

Dowex 1 anion exchange resin was prepared by washing first with 3 N hydrochloric acid until free of impurities absorbing at 260 $m\mu$, then with

2 M sodium formate until free of chloride ion, and finally with water. Orthophosphate was determined by the method of Fiske and Subbarow (17); acid-labile phosphate was the phosphate liberated after 15 minutes hydrolysis in 1 N sulfuric acid at 100°, and total phosphate included the phosphate liberated by ashing with a sulfuric acid-nitric acid mixture. Pentose was determined by Mejbaum's method (18) with a heating time of 40 minutes and recrystallized ribose and arabinose as standards.

Enzymatic hydrolysis of TPN by purified nucleotide pyrophosphatase (4) was carried out as follows: 26 mg. of TPN were dissolved and neutralized with sodium hydroxide and the volume was adjusted to 3.2 cc. Glycylglycine buffer (0.3 cc., 0.25 M, pH 7.4) and nucleotide pyrophosphatase (0.1 cc., 3500 units per cc., 1665 units per mg. of protein) were added and the mixture incubated at 40°. After 23 minutes less than 2 per cent of the TPN remained. After 26 minutes, 0.15 cc. of 1 M basic lead acetate was added and the mixture was kept at 2° for 20 minutes. The precipitate was removed by centrifugation, washed with four 2 cc. portions of 0.1 M lead acetate and then suspended in 2.0 cc. of 0.4 N acetic acid. The supernatant and washings were pooled and treated with 0.1 cc. of glacial acetic acid. Lead was removed from both fractions with hydrogen sulfide and the lead-free filtrates were aerated and neutralized.

Potato adenosine-5-phosphatase was prepared as follows: 1 kilo of peeled Maine potatoes was homogenized in a Waring blender with 2 volumes of distilled water (in five separate batches). To 2.8 liters of filtrate were added 950 gm. of ammonium sulfate; the precipitate was removed by filtration and to the filtrate (2.5 liters) were added 550 gm. of ammonium sulfate. This precipitate was collected by filtration, dissolved in water, and dialyzed 90 minutes against running, cold distilled water. The dialyzed fraction (61 cc.) was acidified to pH 4.5 with 2.7 cc. of 0.1 N acetic acid and fractionated with 95 per cent ethanol at -5° to -10°. The precipitate obtained with 30 cc. of ethanol was discarded; that obtained after a further addition of 100 cc. was dissolved in 15 cc. of water. This fraction as compared with crude potato extracts was 20 to 50 times more active in splitting adenosine-5-phosphate on a protein basis, but only about 5 times as active in the rate of splitting yeast adenylic acid. At pH 5.0 (0.1 M acetate, 0.01 M magnesium chloride) adenosine-5-phosphate was split only 2.1 times as rapidly as yeast adenylic acid. By carrying out the reaction at an alkaline pH, it was possible to limit the enzymatic action to adenosine-5-phosphate. Thus at pH 9.4 (0.1 M glycine, 0.01 M magnesium chloride) the hydrolysis of adenosine-3-phosphate and adenylic acid was completely inhibited, while about one-third of the adenosine-5-phosphatase activity remained.

Results

Conversion of TPN to Diphosphoadenosine and Nicotinamide Nucleotide—The enzymatic hydrolysis of TPN by nucleotide pyrophosphatase repeatedly produced the constituent mononucleotides in near quantitative yield. The water-insoluble lead salt has been designated as diphosphoadenosine and the water-soluble lead salt as nicotinamide ribose phosphate. The data which support these designations are in Table I.

The diphosphoadenosine fraction contained adenine, organic phosphate, and pentose in a ratio of 1.0:2.0:1.2. The acid-lability of the phosphate is consistent, as previously stated (10), with esterification of one phosphate at carbon 5 and the other as in yeast adenylic acid. A small con-

TABLE I
Conversion of TPN to Diphosphoadenosine and Nicotinamide Nucleotide

Substance estimated	Total reaction mixture		Diphospho-adenosine fraction	Nicotinamide ribose phosphate fraction
	0 min.	23 min.		
	μM	μM	μM	μM
TPN	25.9	0.4		
Phosphate, ortho	0.0	2.8	1.8	0.0
“ acid-labile	10.5	14.6	10.9	0.0
“ total	81.3		52.9	23.1
Pentose			31.8	25.0
“Adenine”*	24.0		25.5	
Nicotinamide-ribose*	29.2		2.7	28.1
Adenosine-5-phosphate*	0.0	0.0	0.0	

* “Adenine” was estimated by light absorption at 260 $m\mu$ with use of a molar extinction coefficient of 16,000, after correction for the nicotinamide absorption. Nicotinamide-ribose moiety was estimated by fluorescence (15) and adenosine-5-phosphate spectrophotometrically with Schmidt's deaminase (19).

tamination of nicotinamide ribose phosphate was present. The lack of any reaction with muscle adenylic acid deaminase indicates both the absence of adenosine-5-phosphate in this fraction and the failure of this enzyme to deaminate the diphosphoadenosine. The nicotinamide ribose phosphate fraction contained nicotinamide-ribose moiety, pentose, and organic phosphate¹ in a ratio of 1.0:0.8:0.9.

¹ Evidence that the phosphate of nicotinamide nucleotide is esterified at carbon 5 has been afforded by studies with a purified bull semen phosphatase (20). Crude seminal plasma and a 50-fold purified preparation split, at comparable rates, adenosine-5-phosphate, inosine-5-phosphate, and nicotinamide nucleotide both from DPN (shown by Schlenk (3) to be nicotinamide ribose-5-phosphate) and from TPN. These enzyme preparations acted on ribose-5-phosphate at a much slower rate. The purified preparation did not hydrolyze adenosine-3-phosphate or adenylic acid α .

Diphosphoadenosine Inactive As Phosphate Acceptor—In view of the close similarity of the diphosphoadenosine fragment from TPN to adenosine-5-phosphate and adenosine pyrophosphate, it was of interest to determine whether it would function like the latter nucleotides as an acceptor of phosphate from phosphopyruvate. Schlenk *et al.* (8) had shown that alkaline hydrolysis of TPN did not, as in the case of DPN, yield a phosphate acceptor in a comparable system. In keeping with their findings we observed no perceptible activity with the isolated diphosphoadenosine. The test to determine activity in accepting phosphate from phosphopyruvate depended on the formation of pyruvate, measured spectrophotometrically by the oxidation of reduced DPN in the presence of purified lactic dehydrogenase (11). The source of transferring enzyme was an ammonium sulfate fraction prepared from an aqueous extract of rabbit muscle. The pyruvate appearance, in a 2.5 minute incubation period, was $0.1 \mu\text{M}$ (micromole) with $0.05 \mu\text{M}$ of adenosine-5-phosphate present, and $<0.002 \mu\text{M}$ with $0.28 \mu\text{M}$ of diphosphoadenosine present. Subsequent addition of adenosine-5-phosphate to the diphosphoadenosine reaction mixture resulted in the same rate of pyruvate production observed with adenosine-5-phosphate alone. With adenosinepyrophosphate, the rate of pyruvate production was approximately 10 times as rapid as with adenosine-5-phosphate.

Conversion of Diphosphoadenosine to Adenosinemonophosphate—It appeared that the structure of diphosphoadenosine could be elucidated if some method were available for specifically removing the phosphate group esterified to carbon 5, thus permitting a comparison of the remaining adenosinemonophosphate with adenosine-3-phosphate and adenylic acid *a* (presumably adenosine-2-phosphate). The phosphate at carbon 5 shows greater stability in acid than the phosphates at positions 2 and 3, and has similar stability in alkali, thus precluding the use of acid or alkaline hydrolysis. In surveying natural sources for the proper enzymatic reagent to carry out this selective hydrolysis, it was observed that semen preparations and snake venoms which were highly active in splitting adenosine-5-phosphate did not attack diphosphoadenosine. Thus, samples of dried venom (*Crotalus adamanteus* and *Agkistrodon mokasen*) which at a level of 0.01 mg. completely hydrolyzed $1 \mu\text{M}$ of adenosine-5-phosphate in 20 minutes at pH 8.5, 38° , released little or no phosphate ($<0.05 \mu\text{M}$) from $1 \mu\text{M}$ of diphosphoadenosine even at a level of 1 mg. Human and bull semen preparations yielded comparable results. Adenosine-5-phosphatase preparations from potato, however, did attack diphosphoadenosine, releasing 50 per cent of the phosphate as a limit and at a rate comparable with its action on adenosine-5-phosphate.

The course of hydrolysis of diphosphoadenosine by the potato enzyme in two experiments is shown in Fig. 1. In a total of four experiments the

phosphate release varied from 92 to 96 per cent of the theoretical value. The adenosinemonophosphate was recovered from the incubation mixtures by coagulating the protein at 100° for 3 minutes, removing the orthophosphate with barium acetate or magnesia mixture, and precipitating the nucleotide as a lead, mercury, or silver salt. While this procedure has resulted in products which were indistinguishable from those obtained by the procedure ultimately adopted, the yields were too low to permit conclusions concerning the identity of the starting material. By the use of Cohn's ion exchange method (13) it was possible to obtain good yields

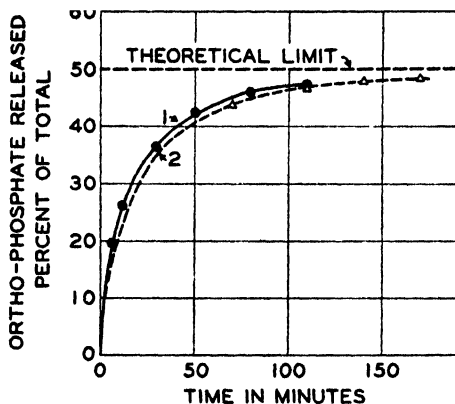


FIG. 1. Hydrolysis of diphosphoadenosine to adenosinemonophosphate by potato adenosine-5-phosphatase. The line drawn at 50 per cent indicates hydrolysis of one-half of the total phosphate. Curves 1 and 2 represent two experiments which are essentially alike. Curve 1, 12 μ M of diphosphoadenosine, 45 μ M of magnesium chloride, 200 μ M of glycine buffer (pH 9.4), and 2.0 cc. of potato enzyme in a final volume of 4.35 cc. Curve 2, 28 μ M of diphosphoadenosine, 60 μ M of magnesium acetate, 250 μ M of glycine buffer (pH 9.4), and 3.0 cc. of potato enzyme in a final volume of 5.45 cc. Incubation at 40°. Aliquots of 0.05 cc. were removed at the indicated time intervals and the concentration of orthophosphate determined.

and at the same time to gain evidence for the identity and homogeneity of the product.

The incubation mixture described in Curve 2, Fig. 1, at 190 minutes was diluted with water and 0.2 cc. of ammonium hydroxide to a volume of 30 cc. This was adsorbed on a column (14 cm. \times 1 sq. cm.) of Dowex 1 (formate) anion exchange resin. Elution was with 0.1 M formic acid at a flow rate of approximately 0.5 cc. per minute. 20 cc. fractions were obtained and their optical density at 260 $m\mu$ determined. The total recovery in the eluate was 92 per cent, of which 70 per cent appeared in a discrete fraction between 480 and 600 cc. of eluant. On the basis of Cohn's data (13), previous trials on this column with known mixtures of adenylic acids,

and of subsequent ion exchange experiments (presented below), this is precisely the region in the elution pattern occupied by adenylic acid *a*. The eluate (120 cc.) was concentrated to 5 cc. by vacuum distillation, frozen, and lyophilized. 7.1 mg. of white powder were obtained which contained the following, in micromoles per mg.:

Adenine (260 m μ absorption)	2.16
Nitrogen (Dumas) (divided by 5)	2.10
Pentose	2.44
Phosphate, organic	2.00
" ortho	0.00

Weight loss on drying at 97° *in vacuo*, 5.6 per cent.

On the basis of adenine content this product was 80.3 per cent pure and represented an over-all yield of 61 per cent from the initial TPN sample. This product is the "adenosinemonophosphate" considered in the following section.

Identity of "Adenosinemonophosphate" with Adenylic Acid a

The adenosinemonophosphate obtained from TPN degradation appeared to be identical with adenylic acid *a* and distinctly different from adenosine-3-phosphate on the basis of three separate criteria: ion exchange chromatography, paper chromatography, and phosphatase action of a potato enzyme preparation.

Ion exchange chromatography as applied in the preceding section to the purification of adenosinemonophosphate was used here as an analytic tool (13). The properties of adenosinemonophosphate, adenylic acid *a*, and adenosine-3-phosphate on the Dowex anion exchange column are compared in Fig. 2. Adenosinemonophosphate and adenylic acid *a* are indistinguishable from each other and are quite unlike adenosine-3-phosphate. The region of fractions 16 to 17 is where adenosine-5-phosphate would appear if present.² The absence of any such component further supports the specificity of the potato preparation in hydrolyzing only the phosphate esterified to carbon 5 of diphosphoadenosine.

Paper chromatography with a solvent system of isoamyl alcohol layered on a 5 per cent aqueous solution of dibasic sodium phosphate was used by Carter (13) for the separation of the two components of yeast adenylic acid. As shown in Fig. 3, *a*, adenosinemonophosphate migrates at the same rate as adenylic acid *a* and when mixed with adenosine-3-phosphate is readily separated from it. While these results indicate the properties of the predominant adenylic acid component of TPN, there

² Personal communication from Dr. W. E. Cohn.

was still the possibility that the preparative procedure had eliminated adenosine-3-phosphate which may have been present. For this reason, aliquots of the enzymatic hydrolysis mixture prior to ion exchange treatment (a stage at which the yield from TPN is near quantitative) were chromatographed on paper with the results shown in Fig. 3, b. There

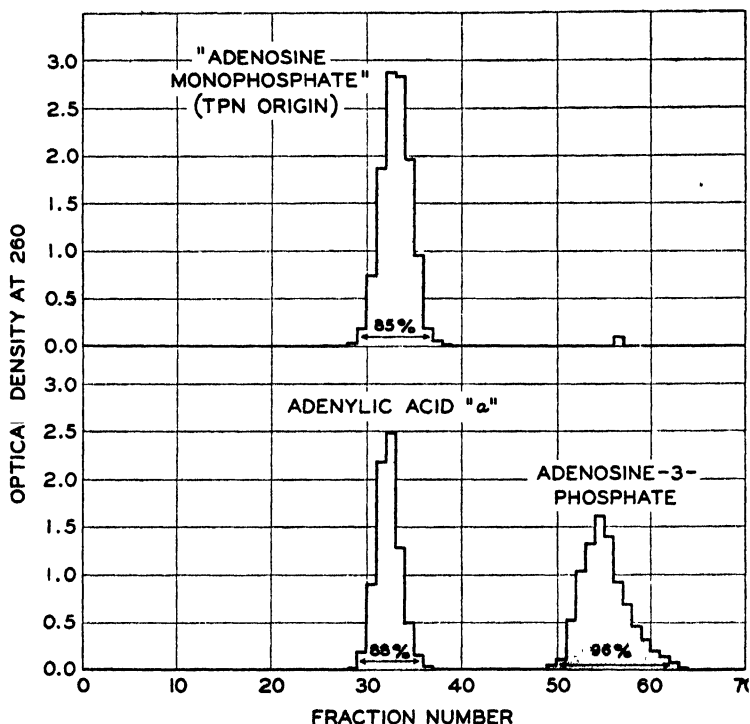


FIG. 2. Ion exchange chromatography of adenylic acids. The resin was Dowex 1 (formate) in a column 13 cm. \times 0.5 sq. cm. The adenylic acids were diluted to 15 cc. with 0.1 N ammonium hydroxide (pH 10.7), adsorbed, washed with 20 cc. of water, and then eluted with 0.1 N formic acid at a flow rate of approximately 0.25 cc. per minute. Fractions were 8.8 cc. The upper elution pattern is for 7.7 μ M of adenosine-monophosphate (see the text) and the lower one is for a mixture of 5.0 μ M of adenylic acid *a* and 5.0 μ M of adenosine-3-phosphate.

was no trace of a component in the area to which adenosine-3-phosphate migrates. The spot in the adenylic acid *a* area was eluted with 0.1 N hydrochloric acid, and on the basis of absorption at 260 μ m represented 91 per cent of the aliquot applied to the paper.

Phosphatase action by a potato enzyme preparation which was more active in splitting adenosine-3-phosphate than adenylic acid *a* provided further evidence for the identity of adenosinemonophosphate with the

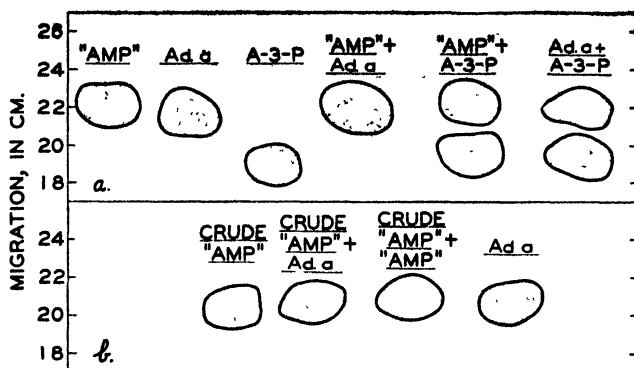


FIG. 3. Paper chromatography of adenylic acids. Approximately $0.15 \mu\text{M}$ of an adenylic acid sample was applied as a spot (1 cm. in diameter) on Whatman No. 1 filter paper. The paper, formed into a cylinder, was placed in a cylindrical jar containing 5 per cent dibasic sodium phosphate with a covering layer of isoamyl alcohol. After 24 hours at room temperature the solvent front had ascended 32 cm. beyond the starting line. The paper was dried at room temperature and examined with a model V41 Mineralight fluorescent lamp. The areas occupied by adenylic acid are sharply defined dark spots. The adenylic acids are designated as follows: AMP = adenosinemonophosphate (see the text), Ad. α = adenylic acid α , A-3-P = adenosine-3-phosphate, and crude AMP = adenosinemonophosphate prior to ion exchange chromatography.

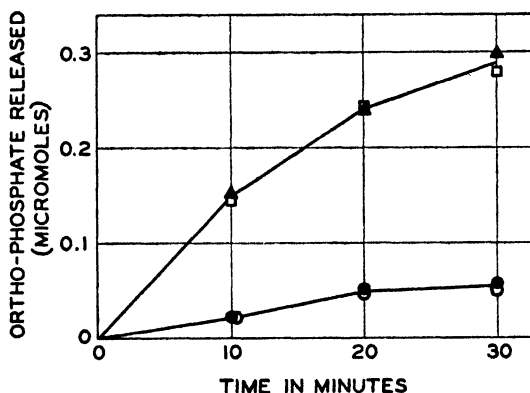


FIG. 4. Phosphatase action on adenylic acids by a potato enzyme preparation. The incubation mixture contained 0.1 cc. of a dialyzed ammonium sulfate fraction (0.015 mg. of protein, see the text), $10 \mu\text{M}$ of magnesium chloride, $100 \mu\text{M}$ of acetate buffer, pH 4.0, and $0.5 \mu\text{M}$ of adenylic acid in a final volume of 1.0 cc. Incubation at 40° . ● adenylic acid α , ○ adenosinemonophosphate, ▲ adenosine-3-phosphate, □ adenosine-3-phosphate + adenosinemonophosphate.

latter. With a preparation obtained by refractionating potato extract with ammonium sulfate between 0.6 and 0.9 saturation,³ the results shown

³ Kindly furnished by Dr. L. A. Heppel.

in Fig. 4 were obtained. Adenosinemonophosphate and adenylic acid α were both hydrolyzed at the same rate, which was only about one-fifth that of adenosine-3-phosphate.

DISCUSSION

Carter and Cohn suggested that the isomerism of the two yeast adenylic acids resides simply in the location of the phosphate groups at positions 2 or 3 of the ribose chain although an α, β isomerism of the glycosidic linkage could not be excluded. The finding that TPN contains adenylic acid α and the demonstration of direct phosphorylation of DPN to TPN by ATP in the presence of a purified yeast enzyme (11) provide further evidence for the view that adenylic acid α is adenosine-2-phosphate. Concerning any direct relationship between TPN and ribonucleic acid, none is apparent at present. The finding of a new isomer of adenylic acid in the TPN molecule may now encourage the reinvestigation of some interesting adenine nucleotide coenzymes for which the structure has not yet been fully established. The diadenosinetetraphosphate of Kiessling and Meyerhof (21) is considered to contain an adenosine-5-phosphate and an adenosine-5'-triphosphate unit cross-linked in an undetermined way. LePage and Umbreit (22) obtained an adenosinetriphosphate from some autotrophic bacteria which was regarded as different from the conventional adenosine-5'-triphosphate isolated from muscle and yeast. On the basis of the acid lability of the pentose phosphate derived from this nucleotide, they concluded that it is adenosine-3'-triphosphate. In view of the acid lability of the phosphate group of adenylic acid α (13), this conclusion must be reconsidered. More recently, Albaum, Ogur, and Hirshfeld (23) studied an adenosinetriphosphate in oat seedlings and in mung beans which also differs in some of its properties from the nucleotide of muscle or yeast origin. It will be of interest to learn the nature of the adenylic acid isomer present in this nucleotide.

SUMMARY

1. TPN was enzymatically degraded by cleavage of the pyrophosphate bond with purified nucleotide pyrophosphatase to yield a diphosphoadenosine fragment which was separated as a lead salt.

2. Phosphate esterified to carbon 5 of the diphosphoadenosine fragment was specifically hydrolyzed by an adenosine-5-phosphatase preparation from potato to yield an adenosinemonophosphate which was then purified by ion exchange chromatography. The over-all yield from TPN was 61 per cent.

3. By the use of ion exchange chromatography, paper chromatography, and the action of a potato phosphatase preparation, the adenosinemono-

phosphate fragment from TPN was shown to be identical with adenylic acid α (presumptively adenosine-2-phosphate) and different from adenosine-3-phosphate.

4. On the basis of available data, TPN may be considered a dinucleotide of nicotinamide ribose-5-phosphate and adenosine-2,5-diphosphate.

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THE SYNTHESIS OF L-ASCORBIC ACID BY THE ALBINO RAT*

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Although a capacity to synthesize ascorbic acid has been observed in a great variety of plants and in all animals studied thus far, with the exception of man, other primates, and the guinea pig, no clear evidence exists concerning the mechanism of biosynthesis (1, 2). Various 6-carbon sugars and sugar acids have been suggested as precursors, but evidence of a direct transformation has been lacking (3-5). Over-all conversion of sucrose to ascorbic acid has been demonstrated in certain excised plant tissues (6).

Since the discovery of accelerated ascorbic acid synthesis in response to various organic compounds (7-10), nerve-depressant drugs such as chloretone and the barbiturates have been widely used to stimulate ascorbic acid synthesis in a variety of experimental animals (11, 12). The increase in stored and urinary ascorbic acid in such animals led to the finding that the tissues of chloretone-treated rats are capable of synthesizing ascorbic acid *in vitro* from a substrate mixture of pyruvate, glyceraldehyde, and hexose diphosphate (10). The mechanism of action of chloretone in stimulating ascorbic acid synthesis is unknown, but a direct conversion of the drug to ascorbic acid is unlikely in view of the fact that many substances of widely divergent structure resemble chloretone in their stimulating effect (8, 9). There has been no proof, however, that fragments of the stimulants do not serve as precursors, even though the weight relationships argue against a direct conversion.

In the present investigation tracer techniques were used to study the biosynthesis of ascorbic acid by chloretone-treated rats of the Wistar strain. It was found that the animals can utilize the carbon atoms of glucose to a major degree for conversion to ascorbic acid. Bicarbonate conversion was only slight. In contrast, the methyl groups of chloretone are not utilized to a measurable extent for ascorbic acid synthesis. Partial degradation of the biosynthetic ascorbic acid indicated further that the conversion of glucose to ascorbic acid proceeded without appreciable differential dilution of radioactivity in positions 1 and 2 compared to the

* This investigation was aided by grants from the Nutrition Foundation, Inc., the United States Public Health Service, and Hoffmann-La Roche, Inc. A report based on a portion of the data given herein was presented before the American Chemical Society in Philadelphia, April 12, 1950.

remaining carbon atoms of the molecule, provided a correction was made for slight CO₂ fixation in positions 3 and 4. This result suggests a direct utilization of the glucose carbon chain for ascorbic acid biosynthesis.

EXPERIMENTAL

Experimental Animals—Male albino rats of the Wistar strain weighing 300 to 350 gm. were used, since in preliminary studies large strain differences had been found in ascorbic acid excretion after chloretone feeding (13). A basal diet of evaporated milk and distilled water was supplied *ad libitum* for several weeks before the final tests were conducted. In the bicarbonate and glucose experiments 45 mg. of chloretone¹ were administered daily for a control period of at least 4 days. The chloretone was given by stomach tube as a homogenate with 1 ml. of evaporated milk. The ascorbic acid excretion of untreated animals was approximately 1 mg. per day, while chloretone-treated rats excreted 25 to 50 mg. per day, as measured by titration with 2,6-dichlorophenolindophenol (14). At the start of the experimental period, the animals used for the bicarbonate and glucose tests received 45 mg. of standard chloretone by stomach tube; the radioactive bicarbonate or glucose was then administered intraperitoneally, dissolved in 1 ml. of distilled water. In the radioactive chloretone tests 45 mg. of labeled chloretone were given as a homogenate in evaporated milk by stomach tube. During the experimental period (23 to 24 hours) the animals had access to evaporated milk and distilled water *ad libitum*.

Measurement of Radioactivity—All materials were assayed for radioactivity in the form of barium carbonate disks mounted on filter paper (15). Respiratory carbon dioxide was absorbed in CO₂-free 2.5 N sodium hydroxide and the radioactive carbonate was precipitated with barium chloride by the procedure of Mackenzie *et al.* (15). Solid samples were converted to barium carbonate by the wet combustion method of Lindenbaum *et al.* (16), with the exception of the 2,4-dinitrophenylosazone of ascorbic acid which required the following treatment for quantitative combustion.

A vacuum gage and a needle valve were inserted between the combustion apparatus and the water pump. A preliminary combustion was then carried out by evacuating the system to 150 mm. of Hg below atmospheric pressure and boiling for 1.5 minutes, the needle valve being used to maintain this pressure. The system was permitted to stand for 5 minutes, evacuated slowly over a period of 10 minutes to full vacuum, and then treated by the regular combustion procedure of Lindenbaum *et al.* (16).

¹ Chlorbutanol, U. S. P., anhydrous, Eimer and Amend, New York.

The barium carbonate disks were counted in a "Q gas" counter,³ all counts being corrected for coincidence losses, background, and self-absorption. Most samples were counted to a "99/100" error (17) of ± 2.58 per cent (10,000 counts registered), and no samples were counted to a "99/100" error of greater than ± 5.25 per cent (2400 counts registered). The over-all precision of the radioactivities reported is estimated to be ± 10 per cent. It was found that within this precision the self-absorption data of Yankwich *et al.* (18) were applicable for use with the "Q gas" counter.

Preparation of Labeled Materials—C¹⁴-Glucose³ labeled uniformly in all positions was prepared photosynthetically (19). Its specific activity (as barium carbonate) was found to be 5.67×10^5 c.p.m. per mg. C¹⁴-Sodium bicarbonate was obtained from Tracerlab⁴ as an aqueous solution containing 1 mm of sodium bicarbonate in 5 ml. of solution. The activity of this solution (as barium carbonate) was found to be 1.71×10^5 c.p.m. per ml. C¹⁴-Methyl-labeled chloretone was synthesized by a Grignard reaction from C¹⁴-methyl iodide⁴ and ethyl trichloroacetate. The specific activity of this material was found to be 1.94×10^5 c.p.m. per mg. Details of the synthesis are presented in the following section.

Synthesis of C¹⁴-Methyl-Labeled Chloretone—Chloretone has been prepared from methyl magnesium halide and ethyl trichloroacetate by the Grignard reaction (20, 21), though without publication of details that could be followed in the present type of investigation. Preparation of labeled chloretone by the direct condensation of acetone and chloroform (22, 23) was investigated but found to be unsuitable for a tracer scale synthesis, primarily because of the low yield.

The Grignard reaction was carried out in a 40 \times 150 mm. test-tube fitted with a water-cooled condenser which in turn was fitted with a drying tube. Magnesium turnings (0.876 gm. or 0.0361 mole) were placed in the test-tube, followed by 4.79 gm. (0.0338 mole) of inactive methyl iodide dissolved in 7.9 ml. of absolute ether. C¹⁴-Methyl iodide⁴ (1.0 mm), having an activity of approximately 1 mc., was dissolved in 20 ml. of absolute ether and added to the reaction mixture. After formation of the Grignard reagent was complete, the test-tube was cooled to 0° in an ice bath and 2.9 gm. (0.0152 mole) of ethyl trichloroacetate, dissolved in 20 ml. of absolute ether, were added in 3 to 5 ml. portions. The addition reaction was allowed to proceed for 10 minutes, and the reaction mixture was then refluxed gently for a period of 5 minutes. The test-tube was cooled immediately to ice bath temperature, the drying tube was removed,

³ Nuclear Instrument and Chemical Corporation, Chicago, Illinois.

³ We are indebted to Dr. W. Z. Hassid for the preparation of the radioglucose used in this study.

⁴ Tracerlab, Boston 10, Massachusetts.

and 10 ml. of a saturated aqueous solution of ammonium chloride were added through the condenser. The ether was removed *in vacuo* (water pump) and the solution remaining in the test-tube was steam-distilled with the receiver immersed in ice water. A forerun of 4 to 5 ml. was discarded and the next 10 ml. of distillate collected, with recovery of practically all of the chloretone. The distillate was allowed to stand for several minutes until the chloretone had crystallized; this crude product was filtered with suction and air-dried on a funnel for 5 minutes. Further drying was effected by pressing the material between filter papers. Purification was achieved by sublimation at 60–70° under a pressure of 20 to 30 mm. of Hg, giving 346 mg. of white crystals and representing a final yield of 11 per cent based on methyl iodide. In pilot runs yields of 10 to 25 per cent based on methyl iodide were obtained. A sample of inactive chloretone synthesized by this method melted at 77–78° and a sample of commercial chloretone¹ before and after resublimation melted at 78–79°. Mixed m.p. 77–79°. Melting points ranging from 77 to 80–82° have been reported for chloretone "hemihydrate" (24), but Cameron and Holly (25) have claimed that this hemihydrate is not a definite compound. Analyses showed that both the "anhydrous" commercial chloretone and the synthesized material had the composition of a hemihydrate.

$C_6H_7Cl \cdot O \cdot \frac{1}{2}H_2O$.	Synthesized.	Calculated.	C 25.76, H 4.32, Cl 57.05
		Found.	" 25.63, " 4.37, " 56.78
	Commercial.	"	" 25.72, " 4.32

In feeding experiments the chloretone prepared by this method but with inactive methyl iodide stimulated the ascorbic acid excretion by Wistar strain rats in a manner similar to the commercial product. The specific activity of the labeled chloretone obtained in this synthesis was 194,000 c.p.m. per mg. (as barium carbonate) and remained unchanged after two successive purifications by sublimation.

Metabolism Apparatus—The type of metabolism apparatus described by Mackenzie *et al.* (15) was used except that the metabolism chamber was designed to allow for convenient separation of urine and feces and for removal of urine collection tubes during the experiment. The metabolism chamber (Fig. 1) was made by E. Machlett and Son, New York.

Collection of Urine, Feces, and Respiratory Carbon Dioxide—Immediately after injection or feeding of a radioactive test substance each rat was transferred to the metabolism apparatus. The urine was collected in 5 ml. of 10 per cent oxalic acid to obtain maximum preservation of the excreted ascorbic acid (13). The collected urine was diluted to 50 ml. with water, and 0.5 ml. aliquots were pipetted directly into combustion tubes (16) and dried *in vacuo* over Drierite for subsequent conversion to

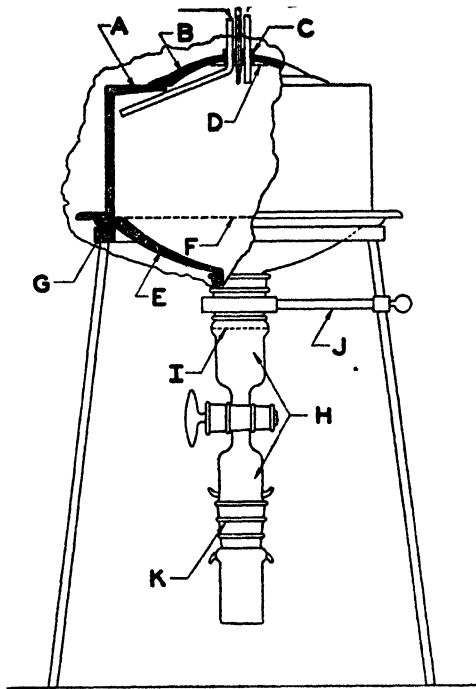


FIG. 1. Metabolism chamber for use in collection of respiratory carbon dioxide, urine, and feces. A, a 250 mm. Scheibler type desiccator, the desiccant portion of which has been cracked off and the resulting surface ground flat. The inverted desiccator is covered with a 6 inch diameter knob type desiccator cover (B), the knob of which has been cracked off and a hole drilled to accommodate a 3-hole No. 6 rubber stopper (C). The rubber stopper mounts an air inlet tube which connects to the source of carbon dioxide-free air in the Mackenzie apparatus (15), a thermometer, an air outlet tube fitted with a T-tube to join the chamber to the flowmeter and manometer of the Mackenzie apparatus, and a zinc wire mesh (D) to keep the test animal from gnawing at the rubber stopper. The inverted desiccator (A) is mounted on an inverted 250 mm. Pyrex desiccator cover (E) provided with a standard taper 55/40 knob, the bottom portion of which is cut off to permit E to serve as a funnel. Support for the test animal is provided by a zinc wire mesh (F), which rests on the shoulder of E. The support disk was coated with clear glyptal and air-dried for 48 hours prior to use. The funnel (E) is connected to a 100 ml. urine collection bottle (K) fitted with a female 45/50 standard taper joint, by means of an adapter (H) which consists of a female 55/50 standard taper joint at the upper end, a 10 mm. bore stop-cock, and a male 45/50 standard taper joint at the lower end. A 2½ inch diameter zinc wire mesh (I) is inserted into the upper portion of H to collect feces. The entire metabolism chamber is mounted on a specially constructed tripod (G) and the adapter (H) is supported by a large condenser clamp (J).

barium carbonate. The feces were dried to constant weight at 100°, pulverized in a mortar, and stored over Drierite *in vacuo*. Respiratory

carbon dioxide was collected and assayed for radioactivity as described by Mackenzie *et al.* (15).

Liver Glycogen—At the end of the experimental period the animal was killed by a blow at the base of the head, decapitated, and exsanguinated, and the liver was then excised and weighed. The liver glycogen was isolated by a combination of the methods of Stetten and Boxer (26) and Good *et al.* (27).

Isolation of Ascorbic Acid As 2,4-Dinitrophenylosazone—Ascorbic acid was isolated from the urine as the 2,4-dinitrophenylosazone by the method which will be described.⁵ Radioactive purity was established as follows: The specific activity remained unchanged after two recrystallizations from 1:1 absolute alcohol-acetone and a third crystallization from acetic acid (by dissolving the osazone in boiling glacial acetic acid, cooling, and pouring into 3 volumes of water). That the relatively high specific activity of ascorbic acid 2,4-dinitrophenylosazone was not due to traces of radioactive glucose 2,4-dinitrophenylosazone was shown by adding to a urine sample 0.635 mg. of labeled glucose with a total activity of 3.6×10^5 c.p.m.; from the mixture inactive ascorbic acid 2,4-dinitrophenylosazone was isolated. The activities per mole of ascorbic acid 2,4-dinitrophenylosazone and of pure L-ascorbic acid were found to be identical when both were isolated from the pooled urine of three rats which had received radioactive glucose.

Isolation of Ascorbic Acid from Rat Urine—Radioactive L-ascorbic acid was isolated from the pooled urine of three chlorotone-treated rats which had been injected intraperitoneally with 20 mg. of labeled glucose per animal. At the end of the 24 hour collection period, total urinary ascorbic acid was found to be 106 mg. by indophenol titration. After the addition of 500 mg. of carrier ascorbic acid the urine was treated with lead acetate and passed through an Amberlite IR-4B anion exchanger.⁵ The acid eluate obtained in this procedure, containing most of the ascorbic acid, was then concentrated *in vacuo* and the ascorbic acid purified by alcohol-ether fractionation and two recrystallizations from a methanol-ether-ligroin mixture. The details of this method have been described (28). The ascorbic acid (140 mg.) was found to be 99.6 per cent pure by indophenol dye titration and melted at 189.5–191° (corrected). Its specific activity was 262 c.p.m. per mg. The radioactive purity was established by the constancy of the specific activity through two successive recrystallizations.

Partial Degradation of Biosynthetic Ascorbic Acid—To measure the radioactivity of carbon 1, the isolated ascorbic acid was decarboxylated. The reaction was carried out by heating a 30 mg. sample at 100° in 20

⁵ Jackel, S. S., Mosbach, E. H., and King, C. G., in preparation.

per cent sulfuric acid under nitrogen for 3 hours. The carbon dioxide was collected in saturated barium hydroxide solution and counted as barium carbonate. Heating periods up to 6 hours resulted in evolution of no more than 1 mole of carbon dioxide per mole of ascorbic acid. The specificity of the method was checked further by using 1-C¹⁴-L-ascorbic acid (29). The specific activity of the barium carbonate obtained by decarboxylating this substance was identical with the specific activity of the 1-carbon of the labeled ascorbic acid per mg. of carbon.

The activity of carbons 1 and 2 combined was obtained by conversion of the ascorbic acid to oxalic and threonic acids by oxidation with sodium hypiodite (30, 31), followed by isolation of the oxalic acid as calcium oxalate monohydrate (32). The calcium oxalate was purified by repre-

TABLE I

Excretion of C¹⁴-Carbon Dioxide after Dosage with C¹⁴-Labeled Glucose, Bicarbonate, and Chloretone

Substance administered	Rat No.	Weight of animal	Per cent of dose excreted as CO ₂ at end of									
			1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	8 hrs.	23 hrs.	24 hrs.	
		<i>gm.</i>										
NaHC ¹⁴ O ₃ *	W-1	332	62.2	76.1	79.3	80.4	81.0			82.8		
	W-2	348	61.8	74.2	77.3	78.4	79.1			81.3		
C ¹⁴ -glucose†	W-3	366	8.2	25.6	38.8	47.7	53.7	57.7	62.0		79.4	
	W-4	352	1.2	6.4	13.9	21.8	31.7	38.6	52.3		79.3	
C ¹⁴ -chloretone‡	W-5	328	0.0	0.0	0.0			0.0			0.0	
	W-6	320						0.0			0.0	

* Dosage, 1.85 mg. of C¹⁴-bicarbonate, 1.88×10^7 c.p.m., by intraperitoneal injection.

† Dosage, Rat W-3, 19.70 mg. of labeled glucose, 1.12×10^7 c.p.m.; Rat W-4 21.17 mg. of glucose, 1.20×10^7 c.p.m., by intraperitoneal injection.

‡ Dosage, 45 mg. of labeled chloretone, 8.76×10^6 c.p.m., by stomach tube.

cipitation and dried at 100° for 1 hour. The radioactivity was determined after conversion to barium carbonate by the wet combustion method (16). The isolated material was shown to be chemically pure by titration with standard permanganate solution.

RESULTS AND DISCUSSION

The data in Table I provide a record of the respiratory C¹⁴-carbon dioxide from chloretone-treated rats after intraperitoneal injection of C¹⁴-bicarbonate and C¹⁴-glucose, respectively. Comparable data for untreated animals which had received methyl-labeled chloretone are also included. The data indicate that glucose oxidation in chloretone-treated rats proceeds at a rate which is essentially similar to that reported for

normal rats (33). The expiration of C^{14} -carbon dioxide following injection of labeled bicarbonate proceeded at a somewhat slower rate than has been reported for normal animals (34). The methyl groups of chloretone were not oxidized to carbon dioxide in detectable quantities within the first 24 hours after administration of the drug.

The data in Table II illustrate the distribution of C^{14} from radioactive glucose and radioactive bicarbonate in the urinary ascorbic acid, expired air, urine, feces, and liver glycogen of chloretone-treated rats. Similar data are shown for the feeding of radioactive chloretone to previously untreated animals. The untreated rats excreted approximately 1 mg. of ascorbic acid per day prior to administration of the labeled drug.

TABLE II

Distribution of Radioactivity after Administration of Labeled Glucose, Bicarbonate, and Chloretone

Substance administered	Rat No.	Urinary ascorbic acid	Per cent of injected dose found in				
			Ascorbic acid	Expired air	Feces	Urine	Liver glycogen
		mg.					
$NaHC^{14}O_3^*$	W-1	40.8	0.02	81.3		1.80	
	W-2	28.0	0.01	82.8		1.42	0.01
C^{14} -glucose†	W-3	41.6	0.27	79.3		3.05	0.13
	W-4	30.5	0.30	79.4	0.17	4.37	0.43
	W-7C	40.8	0.17				
	W-8C	35.4	0.45				
C^{14} -chloretone*	W-5	8.0	0.00	0.00	0.05	11.7	0.00
	W-6	23.8	0.00	0.00		18.7	0.00

* Dosages in Table I.

† Dosages for Rats W-3 and W-4 in Table I. Specimens from Rats W-7C and W-8C represent pooled urines of two and three rats, respectively, each animal receiving radioactive glucose of a dosage similar to that of Rats W-3 and W-4.

It is evident that the carbon atoms of glucose are utilized for ascorbic acid synthesis by the chloretone-treated Wistar rat. The conversion takes place in approximately the same ratio as calculated from the total dietary intake of glucose and the ascorbic acid content of the urine. The slight incorporation of C^{14} from bicarbonate into ascorbic acid probably takes place after conversion to glucose and glycogen. The data exclude the possibility of a direct conversion of the accelerating compound (chloretone) into ascorbic acid during the experimental period. Although this has been verified only for the methyl groups of chloretone, it seems unlikely that either the trichloromethyl carbon or the remaining tertiary carbon would be incorporated into ascorbic acid. Apparently chloretone

acts by accelerating the enzyme system responsible for ascorbic acid synthesis, either directly or by blocking a competing reaction.

Chloretone is also active in stimulating glucuronic acid synthesis in rats (35), with resultant excretion of a not fully identified conjugation product.

The specific activity of urinary ascorbic acid after injection of labeled glucose was approximately 1000 c.p.m. per mg., suggesting that the reactions transforming glucose into ascorbic acid are relatively direct. The biosynthetic ascorbic acid was partially degraded to measure the radioactivity of carbons 1 and 2 separately. The specific activities are given in Table III. The data suggest that the distribution of activity in the 6 carbon atoms of ascorbic acid is essentially identical with that in the initial glucose (which was uniformly labeled in all positions). An exactly uniform distribution of radioactivity in the ascorbic acid derived from uniformly labeled glucose would not, however, be expected in view of the

TABLE III
Degradation of Biosynthetic L-Ascorbic Acid

	Specific activity	Activity	Theory*
	c.p.m. per mg.	per cent of total	per cent of total
Ascorbic acid	262	100	100
Carbon atom 1.....	35.8	14.0	16.7
" atoms 1 and 2.....	78.0	29.8	33.3

* Assuming uniform labeling.

probable contribution of carbon dioxide derived from radioactive glucose and incorporated into ascorbic acid via carbon dioxide fixation. It can be estimated (from the data of Table II) that this contribution amounts to at least 5 per cent of the total activity found in the biosynthetic ascorbic acid. Since it has been shown (36) that biosynthetic glucose synthesized by rats injected with labeled bicarbonate is labeled predominantly in the 3 and 4 positions, it seems reasonable to assume that a similar distribution would hold with respect to ascorbic acid synthesized from carbon dioxide. If it is estimated that 95 per cent of the radioactivity is evenly distributed among the carbon atoms of ascorbic acid and the additional 5 per cent is divided among carbons 3 and 4, it can be calculated that the 1st carbon should contain 15.8 per cent and the first 2 carbons 31.7 per cent of the total activity. These values are in better agreement with the experimental data than those based on a completely uniform distribution of activity.

With this reservation, therefore, it appears likely that the carbon chain

of glucose is not broken before being converted to ascorbic acid, or if so, the fragments are recombined without a major differential dilution effect.

SUMMARY

1. Ascorbic acid synthesis by chloretone-treated rats of the Wistar strain has been investigated by means of radioactive tracer techniques.

2. In the course of this investigation radioactive ascorbic acid was prepared biosynthetically, and methyl-labeled chloretone was synthesized by the Grignard reaction.

3. Following the administration of labeled glucose, approximately 0.3 per cent conversion to ascorbic acid was observed within a period of 24 hours. C^{14} from bicarbonate was recovered in the excreted ascorbic acid to the extent of only 0.01 per cent.

4. Methyl-labeled chloretone did not furnish a measurable quantity of C^{14} in expired carbon dioxide or in excreted ascorbic acid within 23 hours. Only about 15 per cent of the administered dose was recovered in the urine within the same period. Accelerated ascorbic acid synthesis did not proceed via utilization of the stimulating compound as a precursor.

5. The total transfer of C^{14} from glucose to ascorbic acid within 24 hours was approximately equivalent to the total conversion of dietary carbohydrate to ascorbic acid calculated by weight.

6. Partial degradation studies of the biosynthetic ascorbic acid provided evidence that the glucose carbon chain can be utilized for ascorbic acid synthesis, without changing the ratio of activity in positions 1 or 2 to the total activity.

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DEUTEROACETATE IN THE BIOSYNTHESIS OF ERGOSTEROL BY NEUROSPORA*

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The first use of tracers in the investigation of the biosynthesis of sterols was made by Sonderhoff and Thomas (1) in 1937. These workers found that approximately 31 atom per cent deuterium was introduced into the ergosterol of yeast when CD_3COONa was fed. Since this figure was approximately twice as high as the degree of incorporation into the higher fatty acids, and about 20 times as high as the incorporation into the carbohydrates formed by the organism, the conclusion was drawn that the conversion of acetate to sterol does not involve extensive utilization of fatty acids or carbohydrates as intermediates. A similar conclusion was reached by Rittenberg and Schoenheimer (2) in regard to cholesterol synthesis in the rat. Their results indicated that approximately half of the hydrogens of cholesterol are exchangeable at some point in the biosynthesis with the body fluids, and that cholesterol synthesis does not occur by cyclization of fatty acids. Bloch and Rittenberg (3) demonstrated that the deuterium content of rat cholesterol was 3 times as high as the deuterium content of the body fluids when the deuterium was introduced as CD_3COONa . In later experiments (4), these same workers excluded propionic, butyric, succinic, pyruvic, and acetoacetic acids as precursors of cholesterol, and demonstrated, by pyrolysis of the cholesterol obtained, that both the side chain and the steroid nucleus contained deuterium, with a slightly greater amount in the side chain. Further work (5) proved that both of the carbon atoms of the acetate molecule participate in sterol biosynthesis.

The principal sterol of *Neurospora* has been isolated and identified as ergosterol (6). This identification is further supported by the similarity of the infra-red spectra of the isolated sterol and of authentic ergosterol (Fig. 1). An investigation of the rôle of acetate in ergosterol synthesis in *Neurospora crassa* became feasible when an *acetateless* strain was isolated (7). It has been shown¹ that this strain, Y-2492, as the result of mutation is incapable of forming 2-carbon units from sucrose or glucose, and hence supplementation with acetate or ethanol is necessary for its growth. This

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¹ Tatum and Garnjobst, unpublished experiments.

paper will report the results of experiments on ergosterol biosynthesis, with *acetateless* strain Y-2492 and deuterium as a label.

EXPERIMENTAL

With the exception of the growth experiments conducted in heavy water, all cultures were grown in 20 liter Pyrex bottles containing 15 liters of minimal medium (8) with the appropriate supplements. The cultures were incubated with constant aeration with sterile air. The mycelia were filtered off and washed with water. The smaller samples were dried for several hours at 90°, and larger samples were dried at 65° in a forced draft oven. The material was then crushed in a mortar and extracted with acetone in a Soxhlet apparatus for 24 hours. The acetone was distilled

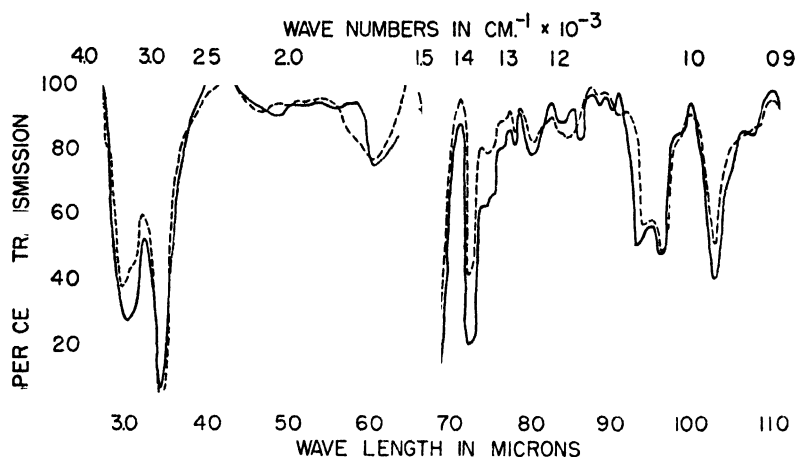


FIG. 1. Comparative infra-red spectra of ergosterol and the principal sterol of *Neurospora*. Ergosterol, solid line; *Neurospora* sterol, dash line.

under reduced pressure and the residue saponified with alcoholic potassium hydroxide for 24 hours at room temperature.

The sterol fraction was removed by ether extraction, and the ether solution was dried over sodium sulfate, filtered, and distilled. The residue was taken up in 90 per cent ethanol and the sterol precipitated with digitonin. The digitonide was burned as such for the deuterium determinations, or was cleaved with pyridine according to the method of Schoenheimer and Dam (9) and the sterol isolated for the infra-red studies. The deuterium concentrations were determined by the falling drop method (10), except in Experiments B and F, in which the sample size necessitated the use of mass spectrometry. All infra-red spectra were determined on a Perkin-Elmer single beam instrument.

The specific growth conditions and the results of the isotopic analyses are listed in Table I. In those cases in which samples of mycelium and fat fraction were removed for deuterium analysis before saponification, appropriate corrections were made in calculating the sterol percentages. The isotope concentrations given in Table I are corrected for the dilution of deuterium by the normal hydrogen present; that is, they represent the per cent conversion of that particular deuterium source into its final form. The acetate used contained 68.9 atom per cent excess deuterium.

TABLE I

Utilization of Deuteroacetate for Fat and Sterol Synthesis in Neurospora
Incubation period of Experiment B 96 hours; all others 120 hours.

Ex- peri- ment	Strain	Sucrose in medium	My- celial weight	Sterol digi- tonide	Per cent sterol	Atom per cent deuterium*		
						Mycelium	Fat	Sterol
15 liters medium containing 6.66 gm. CD ₃ COONa								
A	Wild type	300	64.1	349.8	0.142	0.57 ± 0.03	2.92 ± 0.03	3.22 ± 0.09
B	Mutant	None	2.57	12.0	0.115	9.2 ± 0.3		24.4 ± 1.2
C	"	15	6.86	27.5	0.099	4.5 ± 0.2		22.4 ± 0.4
D	"	300	6.02	22.8	0.136	4.5 ± 0.04	14.0 ± 0.2	17.0 ± 0.6
E†	"	300	17.59	90.1	0.146	1.4 ± 0.03	5.8 ± 0.07	5.8 ± 0.3
0.85 liter acetate-free medium in 11.7 per cent D ₂ O‡								
F	Wild type	17	6.37	28.0	0.108	55.6 ± 1.7		117 ± 9
2 liters medium containing 3.0 gm. of CH ₃ COONa in 5.06 per cent D ₂ O§								
G	Mutant	40	14.26	76.8	0.134	47.4 ± 0.4		68.8 ± 0.6

Atom % deuterium in isolated material

Atom % deuterium in acetate or water of medium $\times 100$.

† Ethyl alcohol added to medium; 30 cc. at time of inoculation, 20 cc. after 24 hours growth, 20 cc. after 48 hours growth.

‡ Incubated in Erlenmeyer flasks (17,125 cc.) on shaker at 25°.

§ Incubated in Erlenmeyer flasks (10,500 cc.) on shaker at 25°.

DISCUSSION

A comparison of the extent of incorporation of deuteroacetate in the wild type strain (Experiment A) and the mutant strain (Experiment D) offers further confirmation of the assumption by Tatum and Garnjobst of a genetic block in acetate formation. Apparently the 2-carbon products of carbohydrate metabolism are essential for the synthesis of much of the cell material.

Since both the wild type and the mutant show approximately propor-

tionally increasing incorporation of acetate in the myelium, fat, and sterol, fat and sterol synthesis in both strains must normally involve acetate, and must be limited to some extent, even in the wild type strain, by the formation of acetate from carbohydrate. This latter assumption is supported by the stimulating effect of acetate on wild type growth.

The data show that ergosterol biosynthesis in *Neurospora* is similar to the synthesis of cholesterol in the rat, since the deuterium concentration of the sterol of the mutant strain on deuteroacetate alone (Experiment B) approached 25 per cent. Comparing the deuterium values obtained with acetate alone (Experiment B) and with limited sucrose (Experiment C), it can be seen that addition of a small amount of sucrose decreases mycelial deuterium by about 50 per cent, but makes only a slight difference in sterol deuterium. Although the mycelial deuterium content is about the same whether the mutant strain is grown in the presence of a low (Experiment C) or a high (Experiment D) concentration of sucrose, the deuterium content of the sterol formed under the former conditions is somewhat higher. In these experiments it was found that the ratio of mycelial weight to acetate concentration is about the same in spite of variations in sugar concentration; hence growth is limited to a large extent by the acetate available. These results prove that acetate is an essential sterol precursor for *Neurospora*. One interpretation of the effects of sugar on sterol deuterium values is that some other compound, derivable from sucrose by this organism, can alternatively supply some of the carbon atoms for sterol biosynthesis. By comparing the sterol deuterium concentrations from the mutant strain grown in the absence of carbohydrate (Experiment B) and in the presence of excess sucrose (Experiment D), it can be seen that this supplementary precursor cannot replace more than about 30 per cent of the acetate.

A comparison of the deuterium concentrations in the mycelia of Experiments D (mutant on acetate and sucrose) and E (mutant on acetate, sucrose, and ethanol) shows a decrease of about two-thirds caused by the addition of ethanol. This is in inverse proportion to the respective mycelial weights in Experiments D and E; hence, in the latter, the ethanol furnished from one-half to two-thirds of the 2-carbon units. Since much more ethanol than acetate was available in Experiment E, these results indicate that acetate is more readily used for sterol synthesis than is ethanol, and suggest that ethanol is oxidized to acetate before utilization for sterol synthesis.

The figure of 117 per cent conversion in the sterol obtained from the wild type strain grown in deuterium oxide (Experiment F) can be explained if two assumptions are made; first, that there is a selection of deuterium by the organism, and second, that, in the metabolism of sucrose to acetate, every carbon-bound hydrogen is exchangeable with the aqueous medium

at some point. With respect to the first assumption, selection of deuterium in favor of hydrogen, although unexpected, is perhaps not too surprising in view of the 100 per cent mass difference involved; rate constants of many reactions are known to be influenced greatly by substitution of deuterium for hydrogen. The second assumption is fully justified when one considers the lability of the hydrogen atoms in the intermediates of the Embden-Meyerhof scheme.

The value of about 69 atom per cent deuterium in the sterol of the mutant strain grown in heavy water (Experiment G) is about what would be expected from the results of other experiments. Considering this value in conjunction with the corresponding values for the wild type strain, and with Experiment B (mutant on acetate alone) in which about 25 per cent of the ergosterol hydrogens were derived from acetate, a value of about 75 atom per cent deuterium would be expected in this sterol.

Infra-Red Spectra—It was thought that the examination of the various deuterated sterols by infra-red spectroscopy might give some information on any differences in the localization of deuterium in the sterol molecule. This examination was undertaken, even though the theoretical calculation of stretching and bending frequencies of certain directed bonds has not been accomplished in the case of the more complex and asymmetric molecules (11). Until recently (12), infra-red spectroscopy, as applied to deuterium-containing organic compounds, has been limited to relatively simple structures ((11) p. 40).

In Fig. 2, the absorption in the neighborhood of 2900 cm^{-1} is due to methyl and methylene absorption principally from the Nujol in which the spectra were determined; unfortunately, this intense Nujol absorption effectively masks any differences which may be present in the methyl and methylene absorption of the samples. The minima at 2350 cm^{-1} are due to atmospheric carbon dioxide absorption. Curve III shows absorption at about 2225 cm^{-1} , corresponding to the carbon-deuterium bond-stretching frequency; Curve IV also shows absorption attributable to carbon-deuterium bond stretching, but in this case the minimum occurs at about 2190 cm^{-1} .

In order to test the reality of these apparent differences in the spectra, the region, 2000 to 2400 cm^{-1} , was examined more closely for fine structure. The samples were dissolved in carbon disulfide for this determination. The curves (Fig. 3) are greatly different in the region of the carbon-deuterium bond-stretching frequencies, the differences almost certainly being due to differences in the location of deuterium in the molecule. The 1375 cm^{-1} methyl deformation frequency (Fig. 4) shows only very slight differences, since the methyl groups in the Nujol are also absorbing in this region.

Substitution of deuterium for hydrogen is known to shift the absorption

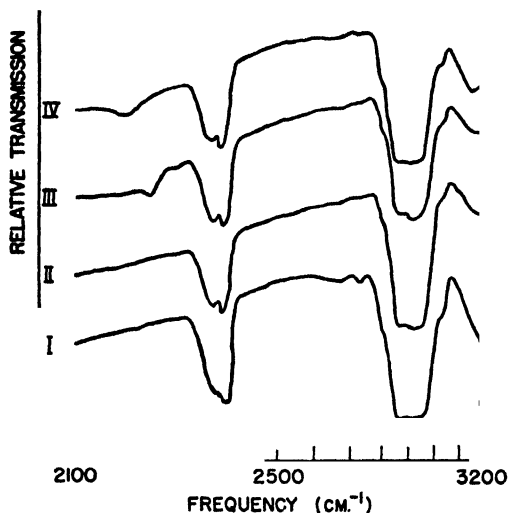


FIG. 2. Infra-red spectra of ergosterol samples in Nujol, 2100 to 3200 cm^{-1} . Curve I, authentic ergosterol; Curve II, ergosterol from *Neurospora*; Curve III, ergosterol from *Neurospora* on deuterioacetate (Experiment C); Curve IV, ergosterol from *Neurospora* on D_2O (Experiment F).

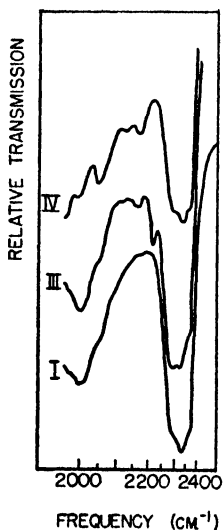


FIG. 3. Infra-red spectra of ergosterol samples in CS_2 , 2000 to 2400 cm^{-1} . For explanation of the curves, see the legend, Fig. 2.

frequencies by approximately $1/1.36$ (13). Significant differences in the spectra are evident in the neighborhood of 1050 cm^{-1} (Fig. 5); although

Curves I, II, and III have an absorption minimum at 1040 cm.^{-1} , the corresponding minimum of Curve IV has been shifted to about 1030 cm.^{-1} . This is the region in which deuteromethyl deformation frequencies would be expected to occur if deuterium were incorporated in any of the methyl groups of the ergosterol molecule.

Analysis of the infra-red spectra, based solely on analogy with much simpler deuterium-containing organic molecules, leads to the tentative conclusion that the ergosterol obtained in Experiment F (wild type strain

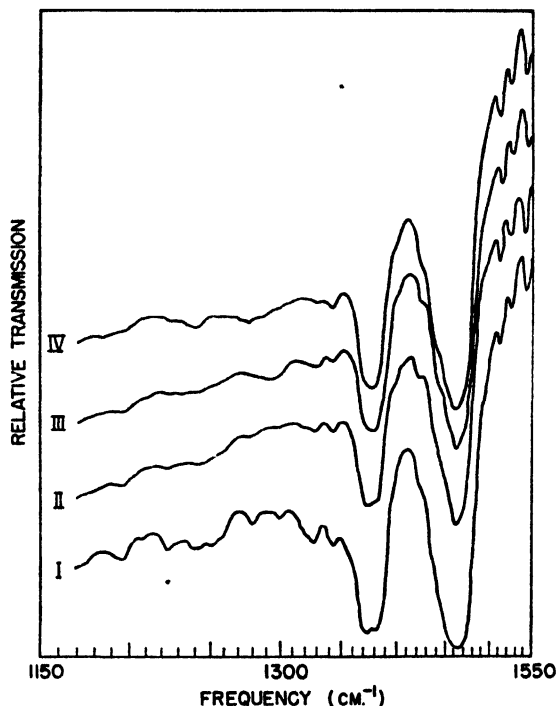


FIG. 4. Infra-red spectra of ergosterol samples in Nujol, 1150 to 1550 cm.^{-1} . For explanation of the curves, see the legend, Fig. 2.

in heavy water, Curve IV) contains a higher concentration of deuterium in the methyl and methylene groups than the ergosterol of Experiment C (mutant strain on deuterioacetate, Curve III). However, in view of the empirical nature of the method, no definite conclusions can be drawn at this time with respect to the location of the deuterium in the ergosterol molecule.

The authors are indebted to Dr. Robert C. Gore of the Stamford Research Laboratories, American Cyanamid Company, and to Dr. Konrad

Dobriner of the Sloan-Kettering Institute for Cancer Research for determination of the infra-red spectra, and for many helpful suggestions in their interpretation. We also wish to express our gratitude to Dr. David Shemin of Columbia University for certain mass spectrometer determinations.

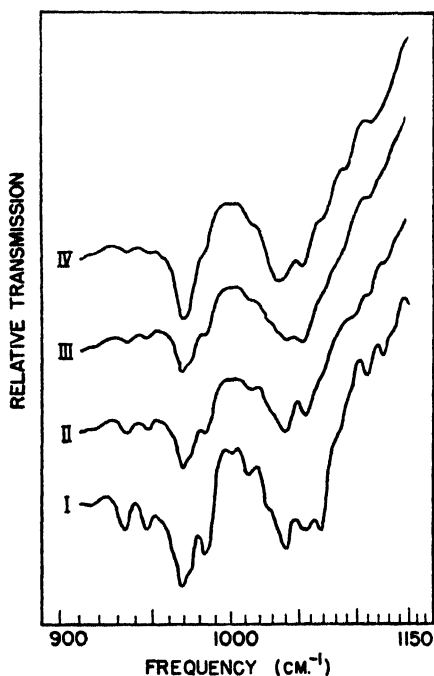


FIG. 5. Infra-red spectra of ergosterol samples in Nujol, 900 to 1150 cm.^{-1} . For explanation of the curves, see the legend, Fig. 2.

SUMMARY

1. The incorporation of deuterioacetate into *Neurospora* mycelium, fat, and sterol has been followed by using the wild type strain and a mutant strain which requires acetate for growth.

2. The results prove that acetate metabolism in the mold resembles that in the rat in that acetate is the principal precursor for fat and sterol synthesis.

3. The infra-red spectra of ergosterol isolated from *Neurospora* grown on deuterioacetate and on normal acetate in heavy water show distinct and different absorption peaks, especially in the region of the carbon-deuterium bond-stretching frequencies, showing that the location of the deuterium in the sterol molecule differs in the two cases.

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SYNTHESIS OF HYDROXYLYSINE

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Hydroxylysine was first recognized as a constituent of protein hydrolysates by Van Slyke and Hiller (1) who in later work (2) established a procedure whereby the relatively pure amino acid could be separated from the lysine fraction by fractional precipitation of the picrates. Van Slyke, Hiller, and MacFadyen (3) studied the reaction of hydroxylysine with periodate in alkaline solution and developed a method for determining hydroxylysine in protein hydrolysates. The periodate reaction, which is specific to vicinal glycols and ethanolamines (4), was shown by Van Slyke, Hiller, MacFadyen, Hastings, and Klemperer (5) to liberate 1 molecule of formaldehyde and 1 molecule of ammonia from hydroxylysine, thus establishing that one of the vicinal ethanolamine carbons was terminal. The influence of the hydroxyl group on the basicity of the amino groups as compared with lysine was studied by Klemperer, Hastings, and Van Slyke (6) and observed to agree with the assumption that the hydroxyl group was vicinal to the more basic (non- α) amino group. These findings established the structure of hydroxylysine as either 2,5-diamino-6-hydroxycaproic acid or as 2,6-diamino-5-hydroxycaproic acid.

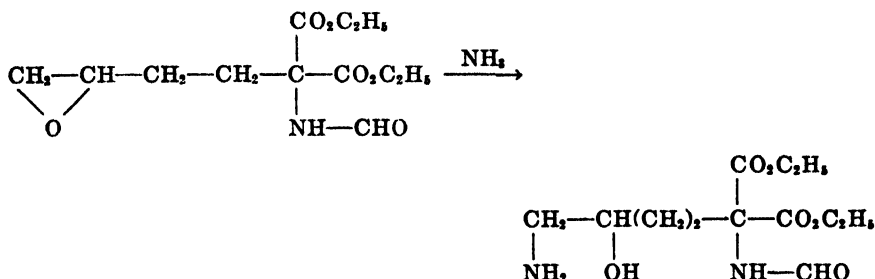
Recently Bergström and Lindstedt (7) have isolated natural hydroxylysine and prepared the dibenzoyl derivative in which they demonstrated the hydroxyl group to be in the 5 position by oxidation with chromic acid. 2 equivalents of chromic acid were utilized to yield 5-ketolysine rather than the 4 equivalents required by the structural isomer to give 2,5-diaminoadipic acid. They also demonstrated the reduction of hydroxylysine to lysine by the action of hydriodic acid and phosphorus. The present work confirms this structure by synthesis of the diastereoisomers and separation of a derivative of the L isomer having identical properties with the same derivative prepared from hydroxy-L-lysine of natural origin.

The introduction of four functional groups into the 6-carbon structure of hydroxylysine requires reactions which do not affect irreversibly any of these groups. The synthesis has been carried out by introducing the hydroxyl and terminal amino groups as a final step under sufficiently mild conditions to restrict secondary reactions. The intermediary com-

pounds can be purified by crystallization or by counter-current distribution, thus avoiding the high temperatures required for distillation.

DISCUSSION

The fundamental reaction in the synthesis is the opening of an epoxide ring by the reaction with ammonia.



Krassousky (8) studied the opening of simpler epoxide rings by ammonia and established that the amine group became attached to the carbon carrying the larger number of hydrogen atoms. Thus both 1,2-epoxy-3-methylbutane and 2,3-epoxy-3-methylpentane reacted with ammonia to give 1-amino-2-hydroxy-3-methylbutane and 2-amino-3-hydroxy-3-methylpentane respectively. The properties of the products were identical with those of the derivatives prepared through methyl magnesium bromide addition to glycine and alanine esters. No evidence of the addition of ammonia to the more substituted carbon atom was observed. Gabriel and Ohle (9) found similar results in the reaction of substituted ethylene oxides with phthalimide. Of the reactions of ethylene oxides with ammonia which have been reported in the literature, no deviations from Krassousky's rule have been reported. The predominant product of the amination reaction is therefore assumed to be the 2,6-diamino-5-hydroxycaproic acid.¹

The presence of 2 asymmetric carbon atoms in hydroxylysine should result in synthesis of four diastereoisomers. The ratio in which they are formed by the present synthesis has not been determined. Stein and Moore (private communication) have studied the rate of appearance of natural and synthetic hydroxylysine hydrochlorides (not carried through the benzoylation procedure but prepared directly from the pic-

¹ Chinard has studied the color developed by the reaction of ninhydrin with amino acids in acid solution and has found that synthetic and natural hydroxylysine give the same color. The nature of this reaction has not been elucidated but appears to depend upon the formation of ninhydrin derivatives of 5- and 6-membered nitrogen heterocyclic rings in which the color of the derivatives is determined by the size of the ring and the substitution with hydroxyl groups.

rate) in the propanol-0.5 N HCl eluate of the starch column (10). Their results revealed identical rates of travel of the two components and absence in the synthetic product of any other components which give color with the ninhydrin reagent. Their findings do not establish the absence of diastereoisomers unless the diastereoisomers could be shown to travel at a significantly different rate.² However, the *N,N'*-dibenzoylhydroxy-DL-lysine and its sodium salt have the properties expected of pure DL compounds. The failure to obtain a crystalline acid from the mother liquor of the sodium *N,N'*-dibenzoylhydroxy-DL-lysine suggests that a mixture of acids is present.

The asymmetric synthesis of the anilide under the influence of papain³ has been used to show the identity of the dibenzoylhydroxy-DL-lysine. The difficult hydrolysis of the product does not result in analytically pure hydroxylysine for comparison of rotation with the natural product. Both natural and synthetic products had low positive rotation but not in satisfactory agreement.⁴ However, the isolation of dibenzoylhydroxy-D-lysine and its sodium salt, which agree in melting point with the L derivatives, and the reconstitution of dibenzoylhydroxy-DL-lysine and its sodium salt from the pure optical enantiomorphs leave no question as to identity. This is further established by preparation of the dibenzoylhydroxy-L-lysine from the hydrolysis product of the synthetic anilide.

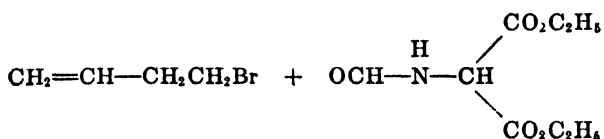
² The rate of travel of any constituent on the starch column is thought to depend upon its distribution coefficient between the mobile and fixed phases as well as adsorptive forces. Under the conditions of the procedure, these forces are but little affected by the presence of other constituents. Thus, in contrast to the determination of melting point and solubility, in which case one or more solid phases are present, optical enantiomorphs travel at the same rate on the starch column. However, as diastereoisomers are different compounds, they should have different physical and chemical properties, although the differences are not ordinarily as great as those of structural isomers. In the present case, one pair of isomers may have been separated in the picrate recrystallization and conversion to the hydroxylysine monohydrochloride, which would explain the absence of asymmetry to be expected with diastereoisomers.

³ Acyl amino acids having the natural L configuration of the α -carbon atoms have been found by Fruton, Irving, and Bergmann (11) to react more rapidly than the optical enantiomorphs with aniline under the enzymatic action of papain. In the present case the purity of the residual dibenzoylhydroxy-D-lysine and of the anilide of the L modification indicates markedly different rates of reaction.

⁴ The analytical methods applied to hydroxylysine have not given satisfactory results. Although impurities which are difficult to remove may be present, the consistency of deviations from theoretical results on many preparations of the picrate and hydrochloride suggests either a lability of the amino acid or that side reactions in the analytical methods do not permit recovery of theoretical amounts of carbon dioxide, ammonia, or nitrogen. The observed positive specific rotations of less than 1 for both natural and synthetic hydroxy-L-lysine in neutral solution must remain tentative until absolute analytical and optical purity can be demonstrated.

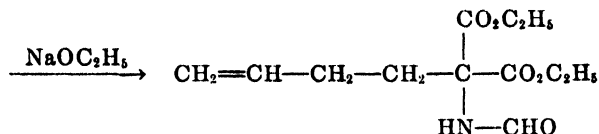
The reactions used are the following.

Reaction I



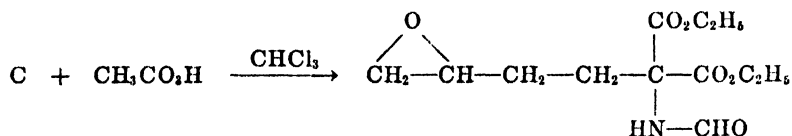
A. Buten-3-yl bromide

B. Ethyl formamidomalonate



C. Ethyl-2-formamido-2-carbethoxyhexen-5-oate

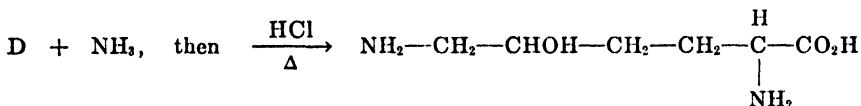
Reaction II



Peracetic acid

D. Ethyl-2-formamido-2-carbethoxy-5,6-epoxycaproate

Reaction III



E. 2,6-diamino-5-hydroxycaproic acid
(hydroxylysine)

Reaction I—The condensation was effected to give a 55 per cent yield of pure crystalline product. Attempted condensation with a commercially available sample of the acetyl rather than the formyl derivative of aminomalononic ester did not result in a crystalline product containing unsaturation. The difference in results with the two acyl derivatives may be attributable to either a difference in steric hindrance to condensation, a difference in inductive effect of the two acyl groups, to some impurity of the commercial material, or to better crystallization of the formyl product from the reaction mixture. Perhaps even better yields with the formyl derivative might have been obtained by slow addition of the sodium ethoxide.

Formamidomalononic ester (B) was prepared by the method of Galat (12). Buten-3-yl bromide (A) was prepared by the procedure of Linstead

and Rydon (13). The purity of this bromide and of the alcohol from which it is formed is essential to a satisfactory synthesis.

Reaction II—Findley, Swern, and Scanlan (14) have studied the conditions for formation of epoxide rings of the D type and for avoiding opening of the rings. The conditions used in the present synthesis are those of Böeseken and Schneider (15) in which the epoxide is formed by reaction with peracid in chloroform solution. The vinyl derivative (C) reacts relatively slowly with peracetic acid, as might be expected of a compound in which two methylene groups separate the vinyl group from strongly inductive groupings. Peracetic acid has several desirable properties. The concentrated chloroform solution required to effect epoxidation at a significant rate can be readily prepared from the commercially available 40 per cent solution in acetic acid. Secondly, the excess of peracid and acetic acid can be partially separated from the final reaction mixture by extraction with water, thus avoiding the local heating effects resulting from extraction with alkali.

To obtain maximum yields of D, it was essential to prolong Reaction II until it approached completion, but not so long that large losses of D could occur from opening of the epoxide ring. The conditions described in the experimental section were designed accordingly. Separation of D from unchanged C was accomplished with the aid of Craig's (16) counter-current distribution procedure with negligible losses. The recovered C could be treated again with peracetic acid.

Reaction III—The epoxide (D) reacts readily with ammonia in alcohol solution. The separation of crystalline hydroxylysine picrate from the reaction mixture was carried out by conventional procedures. Hamilton and Chinard⁶ have found that proline and other secondary α -amino acids liberate carbon dioxide on reaction with ninhydrin subsequent to reaction with nitrous acid, while the primary α -amino acids are deaminated and liberate no carbon dioxide under these conditions. The amount of such secondary α -amino nitrogen and of the difference between the total and primary amino (by nitrous acid reaction) nitrogen in the reaction mixture are nearly equivalent, indicating that little or no disubstitution of ammonia by the epoxide occurs. A sample of the secondary α -amino acid was separated from picrate mother liquors by precipitation with Reinecke salt and converted to the free amino acid. The most probable structure of this contaminant, which could result from solvolysis of the formamido group followed by intramolecular reaction with the epoxide group, is that of the next higher homologue of hydroxyproline (6-membered ring). The picrate of this material is sufficiently insoluble to cause loss of hydroxylysine when the two substances are separated by crystallization of

⁶ Hamilton, P. B., and Chinard, F. P., personal communication.

hydroxylysine picrate. Since this work was done, new ion exchange resins have appeared which greatly facilitate the separation.⁶

Reaction I. Ethyl-2-formamido-2-carbethoxyhexen-5-oate—23 gm. of sodium metal were dissolved in 600 ml. of absolute alcohol. 203 gm. (1 mole) of diethyl formamidomalonate (12) were added, followed by 135 gm. (1 mole) of buten-3-yl bromide (13). The flask was heated gently on the steam cone for 18 hours. After cooling, the now neutral supernatant solution was decanted from the precipitate. The precipitate was partially dissolved in water, and the residue was extracted into chloroform, from which 18 gm. of the desired product separated on concentration. The major portion of the product in the alcoholic supernatant was obtained by removing about half of the alcohol *in vacuo* and adding water to a volume of 500 ml. The crystalline product which readily separated was collected on a Büchner funnel and washed with water. After being dried in air, the material was dissolved in chloroform, dried with sodium sulfate, filtered, and crystallized by slow evaporation of the chloroform. The slightly yellow crystals were washed white on the filter with 15 ml. of water. Combined yield 130 gm. The crystals were colorless and soluble in alcohol, chloroform, and ether. A pure sample was obtained by seeding a concentrated chloroform solution and evaporating the solvent at room temperature until a number of large hexagonal plates had formed. M.p. 84°.

$C_{12}H_{16}NO_6$.	Calculated.	C 56.05,	H 7.45,	N 5.45
	Found.	" 55.83,	" 7.26,	" 5.35

Reaction II. Ethyl-2-formamido-2-carbethoxy-5,6-epoxycaproate—A chloroform solution of peracetic acid free of mineral acid was obtained by mixing 200 ml. of 40 per cent commercial peracetic acid (in glacial acetic acid)⁷ with 100 ml. of chloroform and adding 20 ml. of water to separate the phases. The chloroform layer was separated and the aqueous residue was extracted with four 100 ml. portions of chloroform, thereby reducing the volume of the aqueous-acetic acid phase to 85 ml. The combined chloroform extracts were washed with 10 ml. of water and dried over sodium sulfate. This solution was 1.92 M in peracetic acid determined iodometrically. The total acid, measured by titrating to a phenolphthalein end-point with constant shaking of the phases, was 2.84 M, while to phenol red it was 2.23 M.

500 ml. of this chloroform solution of peracetic acid and 207 gm. (0.80

⁶ At the suggestion of Stein and Moore, the separation of hydroxylysine on a column of the ammonium form of Dowex 50 developed by 0.3 M ammonia has been studied and found to be effective. The natural hydroxylysine used in this study was separated in this way from the basic amino acids of gelatin.

⁷ Supplied by the Buffalo Electro-Chemical Company, Inc., Buffalo 7, New York.

mole) of ethyl-2-formamido-2-carbethoxyhexen-5-oate dissolved in 200 ml. of chloroform were mixed and allowed to react in the ice chest at 4°. The reaction was followed by iodometric determination of residual peracetic acid and by epoxide analysis of the products by the method of Nicolet and Poulter (17). The reaction had slowed to such an extent after 3 days that the solution was allowed to warm to room temperature overnight. Nearly the theoretical amount of peracetic had been used, although 20 per cent of unchanged starting material remained, as estimated by measurement of unsaturation with IBr by the method of Hanus (18). To remove acetic acid and residual peracetic acid from the chloroform solution, the latter was first extracted with three 100 ml. portions of water, in which the acids are preferentially distributed. Finally sufficient 5 N sodium hydroxide was added drop by drop to a fourth 100 ml. of water above the chloroform to extract the last traces of acid and render the aqueous phase alkaline to phenolphthalein. The chloroform phase was further washed with distilled water, separated, dried over sodium sulfate, and concentrated to dryness *in vacuo*. Crystals suddenly formed from the residue on cooling. Residual chloroform was removed in a vacuum desiccator. Yield 215 gm. Determination of epoxide by the method of Nicolet and Poulter (17) and of unsaturation by the method of Hanus (18) showed that the mixture consisted of 73 per cent epoxide and 18 per cent of original hexenoate. A sample prepared after the reaction had proceeded for 3 days at 4° contained 54 per cent epoxide and 37 per cent hexenoate. The IBr reagent of Hanus has not been found to react quantitatively with this hexenoate.

To separate the epoxide from residual hexenoate, the counter-current distribution procedure, the theory and practice of which have been described by Craig *et al.* (16), was applied with benzene and a 50:50 methanol-water mixture as the solvents. The distribution coefficients are approximately 12 for the hexenoate and 2 for the epoxide. 85 gm. of the mixture of products were distributed by the Craig method through four funnels each containing 390 ml. of the methanol-water phase and 80 ml. of the benzene phase. The first benzene and the last methanol-water phases were then separated as "high hexenoate" and "high epoxide" respectively, and the distribution continued by removing pure material from end phases and replacing with fresh phases until further distribution was impractical. The epoxide was obtained in pure crystalline form by concentrating the methanol-water phases *in vacuo* until crystals had formed. 46 gm. of pure epoxide were obtained in this manner. A further 4.2 gm. were obtained by extracting the mother liquor with chloroform and removing the chloroform *in vacuo*. M.p. 75°.

$C_{12}H_{19}NO_4$. Calculated, C 52.80, H 7.01; found, C 52.77, H 6.96

The recovered hexenoate was retreated with peracetic acid. The losses by this procedure were minor.

Reaction III. (Hydroxylysine)—82 gm. of the purified epoxide were dissolved in 200 ml. of 95 per cent alcohol and 300 ml. of 28 per cent ammonia were added. The suspension was warmed gently until solution was complete. After 2 days at room temperature, the solution was concentrated *in vacuo* to remove excess ammonia. The concentration was repeated with the addition of 200 ml. of alcohol to reduce further the concentration of free ammonia. To the 250 ml. volume of concentrated reaction mixture were added 250 ml. of concentrated hydrochloric acid, and the solution was refluxed for 20 hours. The solution was concentrated to dryness. Water was then added and the solution concentrated *in vacuo* three times successively to remove excess hydrochloric acid. 77 gm. of silver oxide were added and stirred to remove chloride ion. The precipitate of silver chloride was removed by centrifugation and washed with water. The washings and supernatant were concentrated *in vacuo* to remove ammonia. An additional 45 gm. of silver oxide were required to obtain a chloride-free solution. The excess of silver ion was removed by precipitation with hydrogen sulfide. The filtered solution, concentrated to remove H_2S , was made up to a volume of 500 ml. and analyzed for total nitrogen by the Kjeldahl method (19), nitrogen liberated by nitrous acid (20), carbon dioxide liberated by the ninhydrin reaction (21), and carbon dioxide liberated by the ninhydrin reaction after previous reaction with nitrous acid⁴ (secondary α -nitrogen of the proline type). Total nitrogen (micro-Kjeldahl), 6.60 gm.; α -amino nitrogen by ninhydrin reaction, 3.59 gm.; primary amino nitrogen by long nitrous acid reaction, 6.065 gm.; secondary α -nitrogen (as determined by an unpublished method of Hamilton and Chinard), 0.473 gm.; secondary and tertiary nitrogen (difference of micro-Kjeldahl and nitrous acid nitrogen), 0.535 gm.

To crystallize the hydroxylysine as picrate, 447 ml. of the chloride-free solution were stirred with 0.195 mole of picric acid and allowed to stand for 1 hour. The suspension was then warmed to 60° for 30 minutes with stirring to complete the solution of picric acid. After standing overnight in the ice chest, the crystals were collected on a Büchner funnel and washed with a small amount of cold distilled water. The picrate was recrystallized from 2 liters of boiling water containing 3 gm. of acid-washed norit. After drying in a vacuum desiccator, the product weighed 46 gm. (0.103 mole). A sample dried *in vacuo* at 100° was analyzed.

	Theory	Found
Amino nitrogen by nitrous acid method.	7.164	7.11
α -Amino nitrogen by ninhydrin method.	3.58	3.49
Nitrogen freed by periodate (3).	3.58	3.45

An additional yield of slightly less pure picrate was obtained by concentrating the mother liquors. A contaminant having the properties of the next higher homologue of hydroxyproline was separated from the picrate mother liquors and purified through the Reinecke salt. This amino acid contained no nitrogen liberated by nitrous acid but 9.7 per cent carboxyl nitrogen.

Hydroxylysine Hydrochloride—30 gm. (7.68 mm) of hydroxylysine picrate were stirred while heating in 50 ml. of N HCl. After cooling, the precipitated picric acid was removed and washed with 10 ml. of N HCl. The picric acid was again heated to boiling with 30 ml. of N HCl, cooled, and again collected. The combined filtrates were extracted with ether and filtered with norit. The clear filtrate was concentrated *in vacuo* to dryness, taken up in distilled water, and stirred with silver oxide until slightly alkaline. The precipitate was collected on the filter and washed with distilled water. The pH of the filtrate was adjusted to approximately 7 by addition of dilute HCl and filtered with norit through a retentive filter paper. The filtrate was concentrated to an oil *in vacuo* and transferred to a 100 ml. beaker with washing. Boiling alcohol was added until the solution became turbid. The oil which separated was rubbed with a glass rod until crystalline. Additional absolute alcohol was added to promote further crystallization. After standing in the ice chest overnight, the crystals were collected and washed with absolute alcohol. Yield 10 gm.; m.p. 225°. Additional material can be recovered from the mother liquors.

$C_6H_{11}N_2O_2Cl$.	Calculated.	N_2 by HONO (20)	14.10,	N_2 by ninhydrin (21)	7.05
	Found.	" " " (20)	13.63,	" " " (21)	6.71

N,N'-Dibenzoylhydroxy-DL-lysine—9.9 gm. (50 mm) of hydroxylysine hydrochloride were dissolved in 10 ml. of 5 N sodium hydroxide and 20 ml. of water. The solution was shaken with 1 ml. of benzoyl chloride and 2.85 ml. of 5 N sodium hydroxide at room temperature. 11.5 ml. of benzoyl chloride in 1 ml. lots, together with proportional amounts of sodium hydroxide, were added in 1 hour. After the seventh addition, the entire volume was filled with crystals of the sodium salt. The reaction mixture was allowed to stand for 3 hours at room temperature to insure hydrolysis of esters. The sodium salt of dibenzoylhydroxy-DL-lysine was collected on the filter and transferred to a beaker in which it was stirred thoroughly with 50 ml. of saturated sodium chloride. The salt was again collected and washed with an additional 50 ml. of saturated sodium chloride in small portions. A sample of the sodium salt recrystallized from absolute alcohol melted at 228–230°. The salt was dissolved in a minimal volume of water, and hydrochloric acid was added to precipitate dibenzoylhydroxy-DL-lysine. Crystallization of the oil occurred readily

when rubbed with a glass rod. The white acid was collected and washed with water. The yield of air-dried material was 13.9 gm. A sample recrystallized from absolute alcohol melted at 139–141°. Additional material can be recovered from the mother liquors after precipitation of the acids, removal of benzoic acid, and reprecipitation as the sodium salt.

Synthetic N,N'-Dibenzoylhydroxy-L-lysine Anilide—13.9 gm. (37 mm) of dibenzoylhydroxy-DL-lysine were added to 28.5 ml. of N sodium hydroxide. Then the extract of 1 gm. of papain with 10 ml. of M citrate buffer at pH 5.2, 0.25 gm. of cysteine hydrochloride, and 1.65 ml. of redistilled aniline were added (11). The volume was made up to 80 ml., the pH was roughly adjusted to 5.2, and the suspension of the sodium dibenzoylhydroxylysine was stirred frequently while standing at 38°. The reaction mixture rapidly became solid as a result of anilide formation. The anilide was collected after 18 hours and washed with water. The precipitate was repeatedly broken up with a glass rod in 0.1 N sodium hydroxide and collected until free from unchanged sodium dibenzoylhydroxylysine, which is strongly held in the precipitate. The precipitate was washed with water, N HCl, and water and dry ether. The precipitate was then dissolved in 250 ml. of hot 80 per cent alcohol and filtered. The filtrate was taken to dryness *in vacuo*; m.p. 223–225°. After washing with 15 ml. of 95 per cent alcohol, the product melted at 230–232°. Yield 4.55 gm.; $[\alpha]_D^{25} = +9.14^\circ$ (82 mg. in 10 ml. of 80 per cent alcohol).

$C_{26}H_{27}N_3O_4$. Calculated, C 70.10, H 6.11; found, C 69.51, H 6.08

Natural Dibenzoylhydroxy-L-lysine and Anilide—Hydroxy-L-lysine hydrochloride of natural origin was similarly converted to the dibenzoyl sodium salt (m.p. 115°) and to the free dibenzoylhydroxylysine (m.p. 172°); $[\alpha]_D^{25} = +3.75^\circ$ (100 mg. in 5 ml. of absolute alcohol).

$C_{20}H_{22}N_2O_5$. Calculated. C 64.85, H 5.96, N 7.54
Found. " 64.45, " 6.01, " 7.43

Reaction with papain and aniline gave the anilide, which melted at 230–232° and was undepressed in melting point on mixing with the synthetic anilide; $[\alpha]_D^{25} = +9.05^\circ \pm 0.50$ (83 mg. in 10 ml. of 80 per cent alcohol).

Dibenzoylhydroxy-D-lysine—The filtrate and washings from the reaction of dibenzoylhydroxy-DL-lysine with aniline and papain were acidified with hydrochloric acid. The crude oil crystallized upon rubbing with a glass rod. After collection and washing with water on the filter, the acid was dissolved in hot alcohol and filtered from the insoluble residue. The solvent was removed on the steam bath and the residue was dissolved in sodium hydroxide, filtered, and acidified. A sample recrystallized from alcohol melted at 172°; $[\alpha]_D^{25} = -3.75^\circ$ (100 mg. in 5 ml. of absolute alcohol). A sample in alcohol was converted to the sodium salt with

5 N NaOH and evaporated to dryness. The salt, recrystallized from alcohol, melted at 115°. Equal weights of the D acid and L acid crystallized together from alcohol melted at 140°, while the sodium salt (from alcohol) melted at 228–230°.

Synthetic Dibenzoylhydroxy-L-lysine—4 gm. of dibenzoylhydroxy-L-lysine anilide prepared from the synthetic amino acid were refluxed with 100 ml. of 95 per cent alcohol and 20 ml. of 6 N HCl until solution was complete. The alcohol was distilled and the residue refluxed with 50 ml. of 6 N HCl for 24 hours. The benzoic acid was removed by ether extraction and the solution taken to dryness *in vacuo*. Silver oxide was added with stirring until the solution was slightly alkaline in reaction. The highly colored solution was extracted with ether, filtered with norit to remove the color, and adjusted to pH 7 with dilute hydrochloric acid. The hydrochloride was precipitated as described above; m.p. 225°.

$C_{21}H_{21}N_2O_5Cl$. Calculated. C 36.35, H 7.63, N 14.10 (21)
Found. " 36.25, " 7.62, " 14.10

450 mg. of this hydrochloride were benzoylated. The alkaline solution was filtered and the residue on the filter washed with water until all the sodium salts had dissolved. The filtrate and washings were acidified with HCl. The aqueous phase was decanted from the precipitate, which was then washed with dry ether. The ether was decanted and the washing repeated. Rubbing the oil under dry ether resulted in crystallization. The crystals were collected and recrystallized from 95 per cent alcohol; m.p. 172–172.5° (micro stage).

The author wishes to thank Dr. D. D. Van Slyke for suggesting the problem and for his constant encouragement and advice. He acknowledges the helpful advice of Dr. Alma Hiller and Dr. Lyman C. Craig. He is indebted to Dr. William H. Stein and Dr. Stanford Moore for the starch column studies, to Dr. Francis P. Chinard for spectral comparisons of the ninhydrin reaction products of synthetic and natural hydroxylysine, and to Mr. D. Rigakos for microanalyses for carbon and hydrogen. He is grateful to the Buffalo Electro-Chemical Company, Inc., for the gift of peracetic acid solution.

SUMMARY

Hydroxylysine has been synthesized.

The *N,N'*-dibenzoyl derivative of hydroxy-DL-lysine has been separated as its crystalline sodium salt.

The anilide of dibenzoylhydroxy-L-lysine has been prepared and shown to be identical with the anilide of synthetic origin.

The identity of dibenzoylhydroxy-DL-lysine has been established by reconstitution from its optical enantiomorphs.

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THE INHIBITION OF D-AMINO ACID OXIDASE BY DESOXYCORTICOSTERONE*

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In the course of investigations in these laboratories on the effect of steroid hormones on various enzymatic systems, it was noted that desoxycorticosterone exerted a greater inhibitory effect than other steroids, and that the D-amino acid oxidase system was particularly strongly affected. This paper deals with experiments elucidating in part the characteristics of the D-amino acid oxidase-desoxycorticosterone inhibition, the effect of other steroids on this enzyme system, and the extent of desoxycorticosterone inhibition on other enzymatic reactions.

Methods and Materials

Preparation of D-Amino Acid Oxidase—The source of D-amino acid oxidase was an acetone powder of pig kidney prepared as follows: Fresh pig kidneys were chilled, deprived of fatty tissue, and ground twice through a medium pore meat grinder. The mince was added to 20 volumes of acetone at -10° , stored for 1 hour in a cold room at 1° , and filtered through a Büchner funnel, and the residue washed with cold acetone. The coarse powder thus obtained was spread out on paper towels and dried in air until all odor of acetone had disappeared. The final drying was carried out in a vacuum desiccator over phosphorus pentoxide. The powder was stored at -10° and a fresh aqueous extract of the enzyme was prepared for use just prior to each experiment. 1 to 2 gm. of acetone powder were ground in a mortar with 15 ml. of ice-cold water and allowed to stand for 10 minutes with stirring. The aqueous extract was centrifuged for 5 minutes to remove any coarse particles and the opaque supernatant fluid added to Warburg flasks containing cold medium and substrates. All operations were carried out in the cold.

An aqueous extract of 80 mg. of this powder, representing about 0.7 mg. of N, took up 180 μ l. of oxygen in the presence of 0.04 M DL-alanine per 60 minutes of incubation. Tissue blanks showed no oxygen uptake. Nitrogen determinations were carried out with the use of a Kjeldahl apparatus.

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Incubation—All incubations were carried out in Warburg flasks at 38° for 60 minutes unless otherwise specified. Final substrate concentrations were as follows unless otherwise stated: DL-alanine, pH 8.5, 0.04 M; sodium pyrophosphate buffer, pH 8.5, 0.017 M; and acetone powder extract containing from 0.4 to 0.8 mg. of N, in a total volume of 3.0 ml. 1 mg. of desoxycorticosterone was added to the main compartment of the Warburg flask in the form of an ether solution and dried in a gentle stream of air. In the experiments comparing the effect of other steroids on D-amino acid oxidase, all steroids, except desoxycorticosterone glucoside, were weighed into the flasks. The glucoside was added as the aqueous solution. 0.1 ml. of a 20 per cent KOH solution was placed in the center wells prior to gassing of the manometers with tank oxygen. All components of the incubation system were present in the center compartments at the start of the incubation unless otherwise specified.

Dialysis and Acetone Precipitation—For the dialysis experiments, 30 ml. of the enzyme extract prepared as described were divided into two portions and incubated with and without 30 mg. of desoxycorticosterone for 30 minutes at 38°. At the end of this time the samples were centrifuged to remove the excess steroid and any other insoluble matter. 5 ml. aliquots were taken from the supernatant fractions and put aside in the cold to be assayed with the dialyzed preparations. The remaining 10 ml. portions were placed in dialysis tubings and dialyzed against 4 liters of distilled water for 18 to 24 hours at 1°. 1 ml. samples were used in the assay.

The enzyme extracts used in the acetone precipitation experiments were prepared as above with the omission of the centrifugation procedure. Instead, the aqueous extracts were strained through two layers of gauze. The presence of coarse particles in the preparation protected the enzyme from inactivation during the precipitation process. The treatment of the enzyme extract with desoxycorticosterone was carried out as described above with the omission of the centrifugation step. 5 ml. aliquots were taken after the 30 minute incubation period, diluted with an equal volume of water, and set aside for assay. The remaining 10 ml. portions were then added to 10 volumes of acetone at -10° and stored in the cold for 2 hours, and the precipitate filtered off rapidly with the aid of suction. The powders thus obtained were dried for a short period in air and then taken up in one-half the original aqueous volume and tested for activity. 1 ml. samples containing 1.0 to 1.4 mg. of N were used in the assay.

Flavin-Adenine Dinucleotide (FAD) and D-Amino Acid Oxidase Apoenzyme—FAD was prepared from fresh beef liver according to the method of Potts.¹ An assay of the preparation used in this study showed the

¹ Potts, A. M., unpublished experiments.

presence of 20 γ of FAD per ml. of solution. The apoenzyme of D-amino acid oxidase was prepared according to the method of Warburg and Christian (1).²

*Source of Steroids*²—Dehydroisoandrosterone hemisuccinate and cholesterol sulfate were prepared in this laboratory by Mr. Frank Unger.

Steroid Analysis—Quantitative analyses of desoxycorticosterone concentrations were carried out according to the method of Corcoran and Page (2) by Miss Dorothy Grim.

Enzymes—The methods used in the preparation and assay of the various enzyme systems are essentially those described in the literature. 1 mg. of desoxycorticosterone was used for every 3 ml. of incubation volume. In every instance the components of the complete enzyme system plus the steroid were together at start of the incubation.

Final molarities for the yeast carboxylase assays were as follows: sodium pyruvate 0.02 M, sodium acetate buffer, pH 5.0, 0.1 M, 10 per cent aqueous bakers' yeast cell suspension 0.5 ml., in 3.0 ml. total volume. Gas phase, nitrogen; temperature 38°; incubation time 30 minutes.

The sources of the enzymes were as follows:² acid phosphatase from "polidase-S," amylase from "rhozyme-DX," L-amino acid oxidase from the venom of *Agkistrodon mokasen*, arginase from beef liver, adenosinetriphosphatase from rat liver, cytochrome oxidase from rat brain homogenate residue and rat liver, pepsin was a commercial preparation, protease from "protease," ribonuclease was the crystalline enzyme, succinic dehydrogenase from rat liver, and xanthine oxidase from beef liver.

EXPERIMENTAL

Effect of Desoxycorticosterone Concentrations—Fig. 1 illustrates the effect of increasing concentrations of desoxycorticosterone on the D-amino acid oxidase system. Depression of the enzyme activity occurred progressively with the steroid concentration. In the presence of 0.7 mg. of tissue N, an inhibition of 82 per cent occurred at the level of 1 mg. of steroid per 3.0 ml. of incubation medium, or an approximate molarity of 0.001. An experiment carried out to determine the actual solubility of desoxycorticosterone in saline at 38° showed that only 63.3 γ went into 3.0 ml. of

² The authors acknowledge the following individuals and commercial establishments for generous gifts of preparations used in this study: Dr. A. M. Potts for the apoenzyme of D-amino acid oxidase, Dr. T. P. Singer for the snake venom, Mr. W. Marsh for the xanthine oxidase preparation, the Rohm and Haas Company for "polidase-S," "rhozyme-DX," and "protease," Ciba Pharmaceutical Products, Inc., for most of the steroids used in this study, Dr. E. C. Kendall for 11-dehydrocorticosterone and corticosterone, Merck and Company, Inc., for 17-hydroxy-11-dehydrocorticosterone, Endo Products, Inc., for all estrogen preparations, and Sharp and Dohme, Inc., for 21-chloroprogesterone.

solution even after 4 hours of shaking. This concentration level suppressed the oxidation of alanine less than 10 per cent. It thus appears that the presence of some component or components of the enzyme system must increase the solubility of desoxycorticosterone.

Effect of Enzyme Concentrations—The extent of the D-amino acid oxidase inhibition in the presence of 1 mg. of desoxycorticosterone varied with the concentration of enzyme present (Fig. 2). When tissue levels of less than 0.4 mg. of N were used, the oxygen uptake was suppressed completely. At higher N concentrations less inhibition was noted. The level of tissue N was kept between 0.4 to 0.8 mg. throughout these experiments.

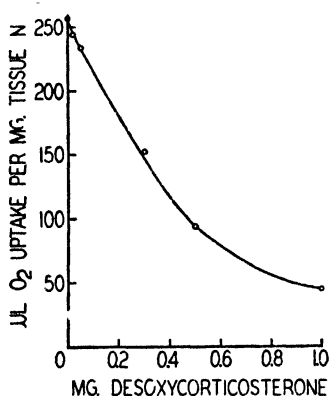


FIG. 1

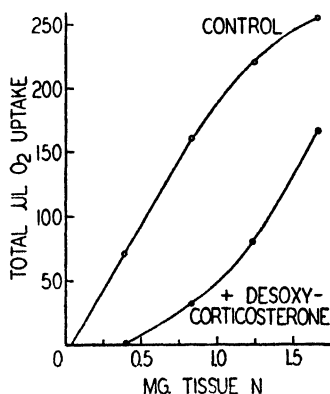


FIG. 2

FIG. 1. The effect of desoxycorticosterone concentration on the activity of D-amino acid oxidase.

FIG. 2. The effect of enzyme concentration on the inhibition of D-amino acid oxidase by desoxycorticosterone.

Effect of Incubation Time—Fig. 3 illustrates a typical picture of the oxygen uptake by D-amino acid oxidase with and without the presence of desoxycorticosterone, when all components of the incubation system are mixed prior to the time when the flasks are placed in the 38° bath. Some oxidation takes place in the first 20 minutes in the presence of desoxycorticosterone. Beyond this point the enzyme is almost completely inactivated. When the enzyme preparation is incubated with desoxycorticosterone 5 to 10 minutes before the addition of the substrate, there is a complete suppression of activity. The inhibition is not relieved by continued incubation.

Effect of Alanine Concentrations—DL-Alanine at 0.04 M (representing 0.02 M D-alanine) was used throughout the study unless otherwise indi-

cated. At this level, 49 to 100 per cent inhibition of activity occurred when all the components of the incubation system plus 1 mg. of desoxycorticosterone were present at the start of incubation. Higher concentrations of alanine protected the enzyme from inhibition under these conditions (Fig. 4, Curve A). The presence of 0.4 M DL-alanine protected the enzyme almost entirely. When the enzyme, desoxycorticosterone, and the usual concentration of alanine were incubated 15 minutes at 38° before tipping in additional amino acid, essentially no

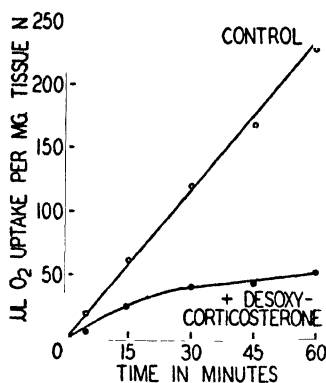


FIG. 3

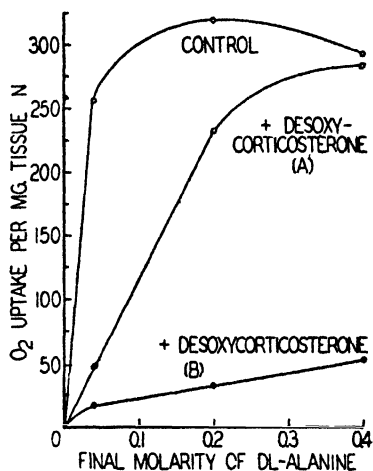


FIG. 4

FIG. 3. The effect of incubation time on the inhibition of D-amino acid oxidase by desoxycorticosterone.

FIG. 4. The effect of alanine concentration on the inhibition of D-amino acid oxidase by desoxycorticosterone. Curve A, alanine concentrations as indicated on the abscissa present in center compartment at zero time. Curve B, 0.04 M DL-alanine present at zero time. Additional alanine to equal final molarities as indicated along the abscissa was tipped in from the side arm 15 minutes after the start of the incubation. O₂ uptake measurements were taken from the time of this addition.

relieving of the inhibition or protection of the enzyme occurred (Fig. 4, Curve B).

The oxidation of DL-isoleucine and DL-methionine by D-amino acid oxidase was also inhibited (about 80 per cent) in the presence of desoxycorticosterone.

Effect of FAD Concentrations—The prosthetic group of D-amino acid oxidase is isalloxazine adenine dinucleotide (FAD) (1, 3). Experiments were carried out to determine whether additional FAD would relieve the inhibition established by desoxycorticosterone. Up to 0.015 μ M of FAD added after 30 minutes of incubation relieved the inhibition only about

6 per cent (Fig. 5, Curve B). The presence of $0.0025 \mu\text{M}$ of FAD before the start of incubation partially protected the enzyme from inactivation (Fig. 5, Curve A). Higher FAD concentrations were of no additional value.

Site of Desoxycorticosterone Inhibition—The above experiments have shown that the inhibition of D-amino acid oxidase by desoxycorticosterone does not appear to be one of a substrate or prosthetic group competitive type. There has been, on the other hand, a number of indications that the inhibition is due to an inactivation of the enzyme itself by the steroid.

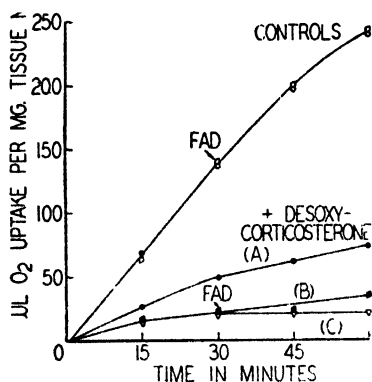


FIG. 5

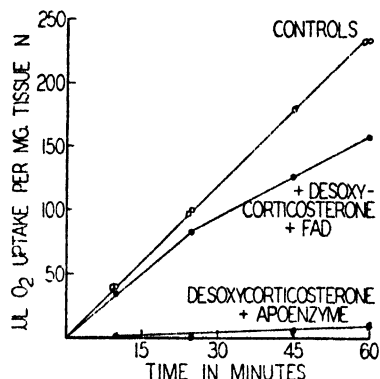


FIG. 6

FIG. 5. The effect of FAD on the inhibition of D-amino acid oxidase by desoxycorticosterone. Curve A, $0.0025 \mu\text{M}$ FAD was present in the center compartment at zero time. Curve B, $0.015 \mu\text{M}$ of FAD was tipped in from the side arm after 30 minutes of incubation. Curve C, no additional FAD.

FIG. 6. The effect of desoxycorticosterone on the coenzyme and apoenzyme of D-amino acid oxidase. Final substrate concentrations, DL-alanine, pH 8.5, 0.4 M ; pyrophosphate buffer, pH 8.5, 0.017 M ; apoenzyme 1 mg. ; FAD $0.025 \mu\text{M}$. 1 mg. of desoxycorticosterone and FAD or apoenzyme were incubated 20 minutes at 38° before the addition of the other components of the complete system.

A series of experiments was conducted to determine the site of inhibition of desoxycorticosterone on D-amino acid oxidase, whether on the prosthetic group or on the protein entity of the enzyme. In one series FAD and desoxycorticosterone were incubated together for 20 minutes before the addition of apoenzyme and alanine, and in the other, the apoenzyme and desoxycorticosterone were incubated together before the addition of FAD and alanine. The fact that high alanine concentrations protect the oxidase from inactivation but do not reverse an inhibition after it is established was used to advantage here. Under these conditions only an inactivation occurring in the incubation of the desoxycorticosterone with either the FAD or apoenzyme prior to the completion of the enzyme

system with necessary components, including a high excess of alanine, should show up in the assay. Once the enzyme system is completed, essentially no inactivation reaction between the steroid and enzyme should be possible. The results from these experiments are illustrated in Fig. 6. The treatment of the steroid with FAD resulted in only a 30 per cent depression of oxygen uptake, while that with the apoenzyme was almost completely inhibited. It thus appears that the desoxycorticosterone reacts with the protein entity of the D-amino acid oxidase to destroy its action.

Reversibility of Desoxycorticosterone Inhibition—The nature of the desoxycorticosterone-D-amino acid oxidase inhibition was next investigated. A dialysis of an inhibited enzyme preparation at 1°, in a volume of water sufficient to dissolve the steroid present, resulted in no relieving of the inhibition. On the other hand, precipitation of the inhibited enzyme with acetone resulted in a regeneration of the oxidase activity (Table I). It

TABLE I

. *Effect of Dialysis and Acetone Precipitation on Activity of Desoxycorticosterone-Inhibited D-Amino Acid Oxidase*

		Non-treated preparation		Treated preparation	
		$\mu\text{l. O}_2$ per mg. N per hr.	per cent change	$\mu\text{l. O}_2$ per mg. N per hr.	Per cent change
Dialysis	Control	188		144	
	Inhibited enzyme	74	-61	54	-63
Acetone pre- cipitation	Control	132		133	
	Inhibited enzyme	63	-52	125	-6

thus appears that the attachment of the steroid to the protein is one which cannot be easily disrupted by dilution with aqueous media, but one which is susceptible to treatment with an organic solvent such as acetone. Further studies on the nature of steroid-protein complexes are now in progress. Of interest is the fact that the reaction of desoxycorticosterone and D-amino acid oxidase is such that the ability of the enzyme to oxidize a substrate is not destroyed.

Comparative Inhibitory Effects of Various Steroids on D-Amino Acid Oxidase—A total of thirty-two steroids was studied for their ability to inhibit the D-amino acid oxidase system (Table II). The comparative activity observed is evaluated as the ratio of the inhibition produced by a specific steroid to the inhibition by 1 mg. of desoxycorticosterone run simultaneously. A ratio of 1 indicates an activity equal to desoxycorticosterone, a value less than 1 a lower relative activity, and a value above 1 a greater activity.

The results obtained show that desoxycorticosterone alone in the group of essentially water-insoluble steroids was capable of exerting a strong inhibitory effect on D-amino acid oxidase. More soluble derivatives and compounds similar in structure to desoxycorticosterone were less active or completely inactive. Desoxycorticosterone acetate, desoxycorticosterone glucoside, progesterone, and 17-hydroxy-11-dehydrocorticosterone were definitely less effective. Corticosterone, 11-dehydrocorticosterone, 17-hydroxycorticosterone, allopregnanetriol-3(β), 17(α), 21-one-20, 21-chloroprogesterone, and ethynyltestosterone exerted no inhibitory action on the D-amino acid oxidase system.

TABLE II

Effect of Various Steroids on D-Amino Acid Oxidase

The range of inhibitions of the desoxycorticosterone controls run simultaneously were from -49 to -100 per cent.

Steroid	Concentration	Ratio*
	mg.	
Sodium androsterone sulfate	1.0	0.65
	2.0	0.88
“ dehydroisoandrosterone sulfate	1.0	0.65
	2.0	0.94
“ “ hemisuccinate	1.0	0.59
“ equilin sulfate	1.0	1.34
	2.0	1.56
“ estrone “	1.0	1.20
	2.0	1.23
“ estradiol sulfate	1.0	1.00
	2.0	1.21
Progesterone	1.0	0.45
Desoxycorticosterone acetate	1.0	0.20
“ glucoside	1.0	0.38
17-Hydroxy-11-dehydrocorticosterone	1.0	0.40

* Ratio $\frac{\text{inhibition produced by steroid}}{\text{inhibition produced by 1 mg. desoxycorticosterone}}$

Other insoluble steroids not effective against the oxidase activity were found to be inhibitory only in a more soluble state or after a prolonged exposure of the enzyme to the steroid in the absence of the substrate. The latter is illustrated in Fig. 7 where testosterone was allowed to react with the oxidase for varying periods of time before the addition of alanine. While only a 19 per cent suppression of activity was observed when the system was complete at zero time, a 53 per cent inhibition level was reached when the steroid and enzyme were preincubated for 30 minutes at 38°. The reason for the decreased per cent inhibition in the flasks preincubated longer than 30 minutes is not apparent.

The androgens tested include androstenediol-3(α),17(α), Δ^4 -androstenedione-3,17, Δ^5 -androstenediol-3(β),17(α), dehydroisoandrosterone, 17(α)-hydroxyprogesterone, methyltestosterone, and testosterone. None of these showed significant inhibitory effects as the free compounds. On the other hand, the highly water-soluble derivatives, sodium androsterone sulfate, sodium dehydroisoandrosterone sulfate, and sodium dehydroisoandrosterone hemisuccinate gave significant inhibitions at the 1 mg. level. Free dehydroisoandrosterone in contrast to its strong inhibition of the oxygen uptake of rat tissue slices (4) had no effect on this system. The findings with the estrogens were similar to those of the androgens. Whereas the free compounds estradiol, estrone, and equilin were without effect, their sodium sulfate esters were as active as desoxycorticosterone.

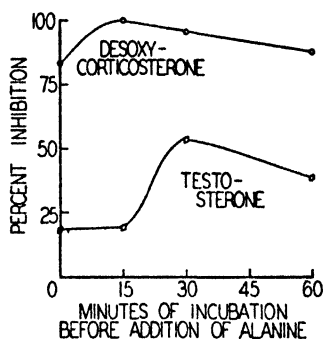


FIG. 7. The effect of prior incubation of testosterone and desoxycorticosterone with D-amino acid oxidase before the addition of substrates. Desoxycorticosterone 1 mg., testosterone 1 mg.; alanine was tipped in from the side arm at the times indicated on the abscissa.

None of the following hormonally inactive steroids exhibited any inhibitory action: cholesterol, sodium cholesterol sulfate, 20,21-epoxy- Δ^4 -pregnenediolone-3, pregnanediol-3(α),20(α), and Δ^4 -pregnenediol-17,20-one-3.

Comparative Influence of Desoxycorticosterone on Various Enzyme Systems—The activities of a number of enzymes were investigated in the presence of desoxycorticosterone to determine whether other systems were as readily inhibited by the steroid as was D-amino acid oxidase (Table III). In every instance a predetermined minimum assayable quantity of enzyme was used with 1 mg. of the steroid per 3.0 ml. of incubation volume to allow a maximum action between the enzyme and inhibitor. Under these conditions D-amino acid oxidase (5) and tyrosinase (6) were strongly inhibited, and urease (7), ascorbic acid oxidase (8), lipase (9), and transaminase (10) were partially inhibited. The activities of three enzyme

systems were increased in the presence of desoxycorticosterone: the action of glutaminase (11), the decarboxylation of pyruvate by whole yeast cells, but not by carboxylase from jack bean meal (12), and the hydrolysis of denatured hemoglobin in the presence of trypsin (13). When casein was substituted for hemoglobin, no stimulation was noted. The following enzyme systems were not affected by desoxycorticosterone: acid phosphatase, amylase, L-amino acid oxidase, arginase, adenosinetriphosphatase, cytochrome oxidase, pepsin, protease, ribonuclease, succinic dehydrogenase, and xanthine oxidase.

TABLE III
Effect of Desoxycorticosterone on Other Enzyme Systems

Enzyme	Source	Substrate	Per cent change in activity, range
D-Amino acid oxidase	Aqueous extract of pig kidney acetone powder	DL-Alanine	-83 (-49 to -100)
Tyrosinase	Aqueous extract of potato peelings	Tyrosine	-98 (-95 to -100)
Urease	Commercial	Urea	-39 (-27 to -50)
Ascorbic acid oxidase	Aqueous extract of squash	Ascorbic acid	-28 (-23 to -32)
Lipase	Commercial	Butyrin	-27 (-25 to -28)
Transaminase	Saline extract of rabbit heart muscle	Glutamic and oxalacetic acids	-21 (-14 to -28)
Glutaminase	Rat and rabbit kidney homogenates	Glutamine	+26 (+12 to +36)
Trypsin	Commercial	Denatured hemo-globin	+73 (+44 to +124)
Yeast carboxylase	Bakers' yeast whole cells	Pyruvic acid	+110 (+106 to +116)

DISCUSSION

Gordan and Elliott (14) and Eisenberg *et al.* (15) have studied the influence of several steroids, with particular reference to desoxycorticosterone on the oxidation of glucose and succinate by rat brain homogenates. Since inhibitions by desoxycorticosterone were not relieved by the addition of methylene blue, they concluded that the activity of a flavin enzyme was suppressed. A similar conclusion was arrived at in this laboratory where a study of the effect of desoxycorticosterone on the oxidation of a variety of substrates by rat tissue preparations was made (4). In every instance a marked suppression of activity occurred, indicating

the probability of an inhibition of the electron transfer system. An assay of the cytochrome *c*-cytochrome oxidase system showed that this was not affected by the steroid. This left the flavin enzymes as the possible site of inhibition. The D-amino acid oxidase system was selected as the model for the elucidation of the nature of the steroid inhibition. The finding that neither xanthine oxidase nor L-amino acid oxidase of snake venom was inhibited under experimental conditions similar to those used for D-amino acid oxidase or that the steroids which suppressed rat tissue oxygen uptake (4) were effective on the D-amino acid oxidase system indicates that the action of desoxycorticosterone on this oxidase is a relatively specific one.

Early in the study it was stated that the concentrations of desoxycorticosterone normally soluble in the volume of the aqueous incubation medium used would only suppress the activity of D-amino acid oxidase some 10 per cent, and therefore that some component of the enzyme system must thereby increase the solubility of the steroid. It now appears that this component is the apoenzyme of the D-amino acid oxidase. It is of interest that certain proteins such as this apoenzyme can draw insoluble compounds such as desoxycort costerone into aqueous solution, and that under certain conditions these compounds can be liberated from the protein with essentially no alteration of the protein structure in so far as can be concluded from the recovery of enzymatic activity observed in the case of the D-amino acid oxidase studied here.

The inhibitory effect of desoxycorticosterone on tyrosinase is of particular interest in view of the impaired tyrosine metabolism observed in patients with Addison's disease.

SUMMARY

1. The inhibition of D-amino acid oxidase by desoxycorticosterone has been demonstrated.

2. High concentrations of the substrate alanine protected the enzyme from inhibition almost completely, but did not relieve an inhibition once it had been established. The same effect was noted with the flavoprotein coenzyme FAD, but to a smaller extent.

3. The inhibition was found to result from a reaction of the steroid with the apoenzyme of D-amino acid oxidase.

4. The inhibition was reversed by an acetone precipitation treatment of the inhibited enzyme with the recovery of essentially all the original enzyme activity.

5. Comparisons of the effect of steroids other than desoxycorticosterone on D-amino acid oxidase, and the effect of desoxycorticosterone on various other enzyme systems are included.

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CORRELATION OF REDUCTIMETRIC AND TURBIDIMETRIC METHODS FOR HYALURONIDASE ASSAY*

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When hyaluronidases act on isolated (as distinct from native) sodium hyaluronate, three changes in the properties of the substrate solution can be measured: (1) the decrease in viscosity, (2) the loss of ability to form insoluble protein salts on acidification, (3) the increase in reducing sugar content.¹

Each of these changes is the basis for a quantitative method of measurement of enzyme activity, and the various methods are all currently employed by workers in the field. The changes listed are often attributed to successive phases of activity of the enzyme on the substrate (1) and the lack of concordance of results obtained with the various methods is sometimes thought to be a reflection of this heterogeneous mechanism. From a chemical standpoint, an interpretation of the mechanism of enzyme action resulting in the increase in reducing sugar is the least ambiguous: the reducing sugar is the result of the breaking of the glucosidic bonds between the alternating *N*-acetylhexosamine and glucuronic acid residues which constitute hyaluronic acid. There is also evidence based on the increase in the color formed with *p*-dimethylaminobenzaldehyde that the first bond which is broken is that which liberates the reducing group of the *N*-acetylhexosamine.

The basis for consideration of different mechanisms has been in large measure due to the fact that the properties both of viscosity and turbidity formation with acidified protein were lost before an increase in reducing sugar could be measured. By application of the recently developed ultramicromethod for reducing sugar of Park and Johnson (2), it is possible to observe that under the conditions employed for turbidimetric assay of hyaluronidase (3), namely the action of 1 turbidimetric unit of testicular enzyme on 200 γ of pure hyaluronic acid in 1.0 ml. of acetate buffer for 30 minutes at 37° and pH 6, reducing sugar equivalent to 1.6 γ of glucose is liberated. This represents 2 per cent of the theoretical

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¹ Mucin clot formation is considered to be a property of native hyaluronate.

quantity to be expected by hydrolytic breakdown of the polysaccharide to disaccharide units.

It therefore seems reasonable to conclude that the chemical reaction which serves as a basis for hyaluronidase measurement by both turbidimetric and viscosimetric methods employing relatively degraded hyaluronic acid is the hydrolysis of glucosidic linkages. This result, which is not an unexpected one, permits a study of the hyaluronidase-hyaluronic acid system at low concentrations of enzyme and substrate by measurement of the products formed, a quantity which is more easily interpretable in a stoichiometric sense than measurement of either viscosity or turbidity formation, which bears an ill defined relationship to the quantity of substrate remaining.

EXPERIMENTAL

Turbidity Reduction Measurements—These experiments were carried out as described by Meyer (3)² with a sample of pure sodium hyaluronate³ as substrate, having the following analysis in per cent: N 2.79, hexosamine 34.9, uronic acid 39.5, sulfate <0.4. This method measures enzyme activity in terms of the turbidity reducing unit (t.r.u.) defined as the quantity of enzyme which, under the conditions described below, in 30 minutes lowers the turbidity formation of 200 γ of substrate to that given by 100 γ .

Reducing Sugar Measurements—Test-tubes containing 200 γ of sodium hyaluronate and 2 to 3 t.r.u. of enzyme in a total volume of 1.0 ml. of 0.1 M acetate buffer, pH 6.0, in 0.15 M sodium chloride,⁴ were incubated at 37°. At 20, 30, and 40 minute intervals, 0.25 ml. aliquots were withdrawn and added to tubes containing 2.75 ml. of water. The reducing sugar in these tubes was then determined according to the directions of Park and Johnson (2). Controls on both enzyme and substrate were found to be unchanged during the course of the experiment. The reducing power of the enzyme and substrate, determined separately, served as the value for zero time in determining the increase in reducing sugar; because these values were low, they were determined in the presence of 1 γ of glucose. All experimental values were obtained in duplicate. In each experiment standards of 1, 2, and 3 γ of glucose in buffer of pH 6 were run in order to establish the reduction equivalent in terms of color produced; the points fell on a straight line in all cases. The standards must be determined in each experiment because of a variation in both

² The concentration of substrate solution was misprinted in Meyer (3) as 4 mg. per ml. and should have read 0.4 mg. per ml.

³ This material was isolated from a mesothelioma (4).

⁴ The pH after sodium chloride addition is 5.9.

the blank and the color production which it has not been possible to eliminate.

Preliminary experiments were run with 1 t.r.u. of enzyme, but when it was found that the increase in reducing sugar was proportional to enzyme concentration (Fig. 1), the quantity of enzyme was raised to about 3 t.r.u. in order to obtain a larger and hence more reliable value for comparison. With 3 t.r.u., the increase was 4 to 5.5 γ of glucose equivalent per ml. in 30 minutes, the value for zero time being 1 to 2 γ of glucose equivalent, in the main contributed by the substrate (0.8 to 1.0 γ).

Smooth curves were drawn through the origin and the points for 20, 30, and 40 minutes (Curve A, Fig. 2). The 30 minute value used to

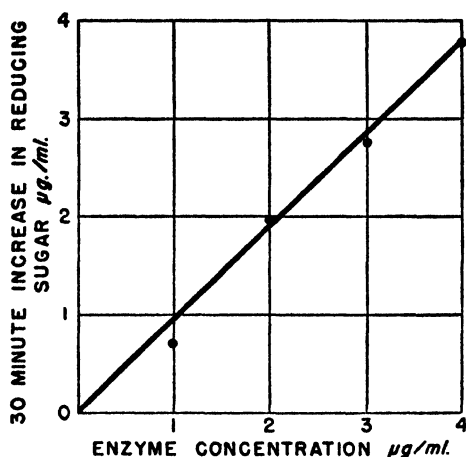


FIG. 1. Enzyme activity plotted as a function of enzyme concentration. 4 γ of enzyme \sim 3 t.r.u.

compare enzyme activity as measured by the turbidimetric method was then obtained from the curve.

To facilitate this comparison, a reducing sugar unit is defined as the quantity of enzyme which produces an increment in reducing sugar equivalent to 1 γ of glucose in 30 minutes at 37° under these conditions, namely, 200 γ of substrate in 1 ml. of acetate buffer at pH 6 containing sodium chloride. The results of such studies carried out with twelve individual testicular enzyme preparations (Table I) show a constancy of the ratio of reducing sugar units to turbidity reducing units within the limits of error of the two methods. The mean value of 1.6 for the ratio r.s.u. to t.r.u. indicates that 1 turbidity reducing unit of enzyme produces reducing sugar equivalent to 1.6 γ of glucose in 30 minutes.

Under these conditions, 2 t.r.u. of enzyme break down the 200 γ of

substrate employed to the point where it no longer produces turbidity with protein. This occurs with the formation of 3.2 γ of glucose equivalent from this substrate.

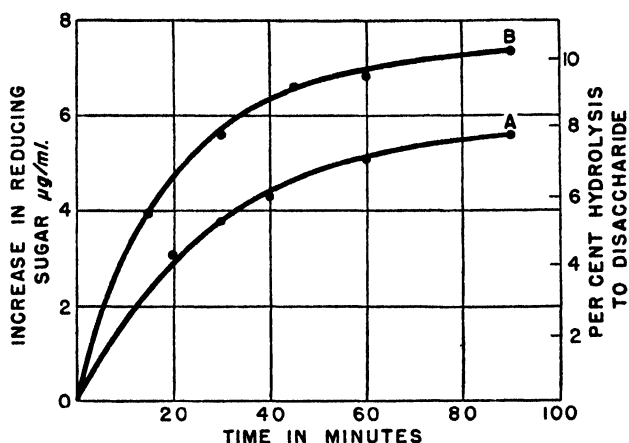


FIG. 2. Course of the reaction. Curve A pH 6; Curve B pH 5. Enzyme concentration \sim 3 t.r.u. per ml.

TABLE I

Comparison of Activity of Testicular Hyaluronidase Preparations by Turbidimetric and Reductimetric Methods

Enzyme preparation No.	Turbidity reducing units per mg.	Reducing sugar units per mg.	$\frac{\text{R.s.u.}}{\text{T.r.u.}}$
1	102	190	1.9
2	105	150	1.4
3	200	290	1.4
4	220	420	1.9
5	230	310	1.3
6	240	340	1.4
7	420	780	1.9
8	450	580	1.3
9	510	880	1.7
10	530	710	1.3
11	590	1120	1.9
12	830	1440	1.7
Mean			1.6

The pH-activity curve (Fig. 3) obtained with phosphate-citrate buffers (0.02 M) shows a broad optimum at values below pH 5.4 with a rather rapid fall in activity at higher values.

The initial reaction velocity as measured by the increase in reducing

sugar in 30 minutes is proportional to the substrate concentration in the range under study (Fig. 4).

The molar reduction equivalents in terms of glucose, determined by this method, of several compounds related to those under study are shown in Table II together with their equivalents determined at higher levels

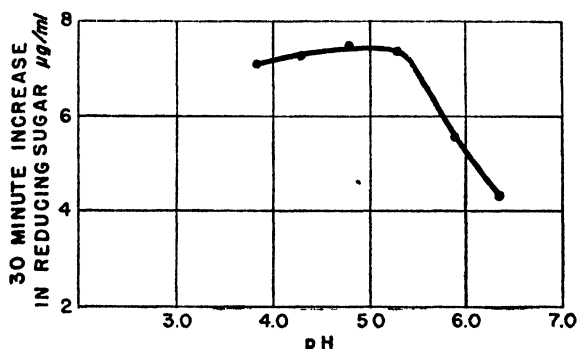


FIG. 3. Enzyme activity plotted as a function of pH. Enzyme concentration ~ 4 t.r.u. per ml. Citrate-phosphate buffers, 0.02 M.

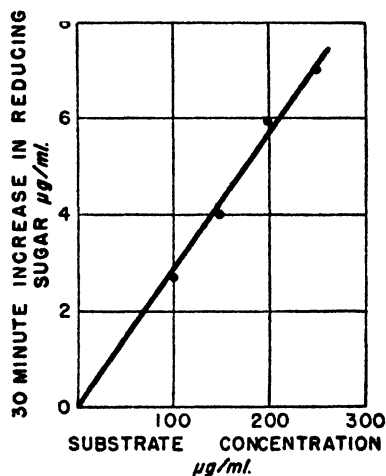


FIG. 4. Reaction velocity plotted as a function of initial substrate concentration, pH 6. Enzyme concentration ~ 3 t.r.u. per ml.

by a modification of the Hagedorn-Jensen method.⁵ It is seen that, while that of glucosamine remains relatively constant, the molar reduction equivalent of *N*-acetylglucosamine increases from 0.81 to 0.97 under

⁵ The reagents and technique of oxidation are those listed in Bates (5). However, the residual ferricyanide is determined colorimetrically by measurement of the absorbance at 4200 Å, as suggested by Schales and Schales (6).

TABLE II

Reduction Equivalents of Compounds Related to Components of Hyaluronic Acid

Compound	Quantity	Glucose equivalent	
		Glucose per gm.	Glucose per mole
Micromethod of Hagedorn-Jensen*			
	γ	gm.	mole
N-Acetylglucosamine	75-150	0.66	0.81
Glucosamine hydrochloride.	75-150	0.88	1.05
Glucuronolactone	50-100	0.98	0.96
Ultramicromethod of Park and Johnson (3)			
N-Acetylglucosamine	1-3	0.79	0.97
Glucosamine hydrochloride.	1-3	0.83	0.99
Glucuronolactone.	1-3	0.79	0.77

* See foot-note 5.

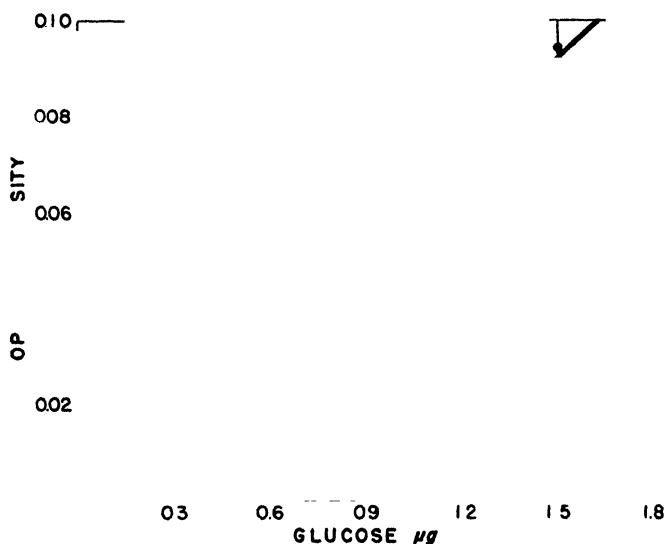


FIG. 5. Relationship of optical density to quantity of glucose in lower range of ultramicromethod.

the influence of cyanide.⁶ The reduction equivalent of glucuronolactone, on the other hand, is adversely affected by cyanide (compared with glu-

⁶ The essential difference between the Hagedorn-Jensen and the ultramicro-procedures is the use of cyanide in the latter to obtain greater sensitivity. The cyanide increases about 3-fold the molar quantity of ferricyanide reduced by glucose.

cose), falling from 0.96 to 0.77. It should be borne in mind that the reducing group liberated in the enzymic reaction is probably closely related to that of *N*-acetylglucosamine.

Park and Johnson report that for samples containing 1 to 9 γ of glucose "the precision of the method is such that over 90 per cent of individual analyses of a known sample fall within 0.2 γ of the expected result." The amounts of reducing sugar measured in the enzyme experiments reported here were between 0.6 and 3 γ . In our experience the method has greater precision than that reported by Park and Johnson, less than 5 per cent of the analyses falling beyond 0.1 γ of the expected result. The color developed is proportional to the concentration of reducing sugar at least down to 0.6 γ (Fig. 5). Occasional individual determinations are, for unexplained reasons, obviously incorrect and must be rejected; when this occurs with the blank, it is revealed by the lack of concordance of the standards.

DISCUSSION

It is recognized that measurements of the formation of reducing sugar are relatively unreliable for the estimation of enzyme activity in crude systems because of the lack of specificity of the oxidizing agents used. For this reason, the turbidimetric and, under some conditions, the viscosimetric methods of hyaluronidase measurement are most certainly those to be preferred when assays of crude enzymes are required. However, in the highly purified system, especially with respect to the substrate, which is under study here, the increase in reducing sugar is a much more reliable criterion of the progress of the reaction than either loss of turbidity formation or drop in viscosity. The reason for this is that interpretations of the kinetic picture obtained with the latter two methods involve the unwarrantable assumption that the products of the reaction do not contribute to the turbidity formation or viscosity.

Studies of the hyaluronidase-hyaluronic acid reaction by means of the increase in reducing sugar involve extensive formation of products even when measured by micromethods. Under such conditions a study of the course of the reaction is complicated by the presence in preparations of testicular hyaluronidase of other enzymes which are known to cause an increase in reducing power due to further breakdown of these products. The measurements carried out by means of the ultramicro-procedure represent a reasonably close approximation to initial reaction velocities and therefore serve to clarify to some extent the reaction kinetics. It can be seen from Fig. 2 that the reaction rate decreases rather rapidly even before any extensive hydrolysis has occurred. At higher levels of enzyme and substrate the same phenomenon is observed.⁷ The

⁷ Unpublished experiments.

explanation for this occurrence may be found in the instability of the enzyme, inhibition of the enzyme by hydrolysis products, or to a difference in the kinetics of breakdown of the products as compared with that of the initial substrate. Analysis of the data obtained under widely different conditions suggests that the major factor is the instability of the enzyme. It is clear that the course of the reaction does not permit a simple kinetic interpretation. Another approach, namely measurement of the initial reaction rate with different initial substrate concentrations, reveals the initial rate to be proportional to substrate concentration, that is, first order with respect to substrate. The inability of Dorfman (7) to demonstrate first order kinetics without the application of a variable correction factor probably results partly from the failure of the course of the reaction to serve as a basis for simple kinetic evaluation and partly from the unjustifiable assumption that the reaction products do not contribute to turbidity formation.

TABLE III

Number of Bonds Hydrolyzed Resulting in Complete Loss of Turbidity Formation Calculated from Assumed Molecular Weights of Substrate

Mol. wt.	No. of repeating disaccharide units	No. of bonds hydrolyzed	Number average mol. wt. of product
25,000	60	3	6300
50,000	120	6	7100
100,000	240	12	7700
200,000	480	24	8000

On the basis of some reasonable, though still to be confirmed, considerations it is possible to interpret the mechanism of the enzymic attack on hyaluronic acid. The loss of turbidity formation with the substrate employed here is obtained with the release of 3.2 γ of reducing sugar as glucose for each 200 γ of the substrate; if hyaluronic acid is composed of a repeating unit of *N*-acetylhexosamine and glucuronic acid, and if the reducing group first liberated has a molar reducing equivalent equal to that of *N*-acetylglucosamine (Table II), then these quantities represent the formation of 0.018 μM of reducing groups from 0.40 μM of repeating units (based on N or hexosamine content), or 4 to 5 per cent hydrolysis of the substrate to the disaccharide. Native hyaluronic acid has been found to be polydisperse with minimal average molecular weight of 200,000 to 500,000 by Blix and Snellman (8). An alkali-degraded product was estimated by them to have a molecular weight of 50,000. For the substrate employed here ($\eta_r = 2.4$ at 37° at a concentration of 0.25 per cent in 0.1 M acetate buffer, in 0.15 M sodium chloride) an average molecular weight of between 25,000 and 200,000 would probably be a

reasonable value. For this range and the per cent hydrolysis calculated, the average molecular weight at which turbidity formation disappears is shown in Table III probably to lie between 6000 and 8000.

The fact that the rupture of relatively few bonds leads to loss of turbidity formation supports the idea that primary enzymic attack occurs in the center of or randomly within the chain, rather than at the terminal periods. A terminal attack would still leave large polymers capable of precipitating with protein even after liberation of many reducing groups.

Turbidimetric experiments with testicular hyaluronidase have generally been carried out at pH 6 (3, 9). This is due to the fact that the original method of Kass and Seastone (10) employed this pH in the study of bacterial enzymes. From the pH-activity curve obtained reductimetrically with testicular enzyme (Fig. 3) it is seen that this pH value is not the optimum and that the activity falls rather rapidly above a pH value of 5.4. Comparison of the results of different laboratories will therefore require more careful consideration of this factor.

The authors wish to acknowledge the valuable assistance of Miss Toby G. Bernstein and Miss Doris A. Hall in this work.

SUMMARY

The activities of twelve individual preparations of testicular hyaluronidase were measured under identical conditions by both turbidimetric and reductimetric methods. It was found that, under these conditions, the "half turbidity" value serving to define the turbidimetric unit is reached with the liberation of 1.6 γ of reducing sugar (as glucose). The two methods are thus shown to measure the same reaction, namely, hydrolysis of glucosidic bonds. From the number of bonds broken which leads to complete loss of turbidity formation, it is concluded that the enzyme attack must occur within the polysaccharide chain rather than at a terminal repeating unit.

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THE OXIDATION OF ACETATE-1-C¹⁴ BY RAT TISSUE IN VITRO*

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There is a considerable body of evidence in the literature to indicate that acetate is oxidized primarily by way of the Krebs cycle, and several previous studies have indicated that homogenates of cancer tissues are deficient in the enzymes that catalyze the oxidation of C₂ fragments (1-4). So far there is little information on the oxidation of acetate *per se* by neoplastic compared with normal tissues. The present work was undertaken to compare acetate oxidation in fortified homogenates (4) with acetate oxidation in tissue slices, by using as a criterion the measurement of C¹⁴O₂ in the center wells of Warburg flasks on incubation of slices or homogenates with acetate-1-C¹⁴, and to correlate the radioactivity data with oxygen uptake under various conditions. These measurements were carried out on homogenates and slices of a variety of normal and neoplastic tissues, including those in which the extra oxygen uptake due to acetate is so small that significant data have heretofore been unavailable. In addition studies involving various intermediates in the Krebs cycle were carried out.

EXPERIMENTAL

Male rats weighing between 200 and 300 gm. were obtained from the Holtzman Rat Company. The rats were killed by decapitation and bled, and the tissues were placed in isotonic KCl at 0° in cracked ice.

Homogenate Experiments—Homogenates were prepared with glass homogenizers, with 9 ml. of cold isotonic KCl per gm. of tissue (4). They were pipetted into Warburg flasks containing essentially the same system which was used to determine oxalacetic oxidase (4), except that acetate-1-C¹⁴ was added and a higher phosphate level was used. Each flask contained the following additions, with the final concentrations

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given in parentheses: 0.4 ml. of 0.5 M KCl (0.067 M), 0.2 ml. of 2×10^{-4} M cytochrome *c* (1.3×10^{-5} M), 0.3 ml. of 0.1 M potassium phosphate at pH 7.1 (0.01 M), 0.1 ml. of 0.1 M MgCl₂ (0.0033 M), 0.3 ml. of 0.01 M K-adenosinetriphosphate (0.001 M), 0.3 ml. of 0.027 M oxalacetic acid (three-fourths neutralized) (0.0027 M), 1 to 10 μ M of sodium acetate-1-C¹⁴, homogenate, and water to make the final volume 3.0 ml. The gas phase was air. In general, the oxygen uptake ceased before 150 minutes, which was the usual duration of the measurements, unless otherwise stated. All values are the average of duplicate flasks.

Slice Experiments—The slices were prepared at 0° with the Stadie slicer (5) and were kept briefly in isotonic KCl at 0° until used. They were put into Warburg flasks containing 3.0 ml. of cold Krebs-Ringer phosphate (6) to which was added sodium acetate-1-C¹⁴. The gas phase was O₂.

General Procedure—With either homogenates or slices, 0.2 ml. of 2.5 N NaOH was placed in the center well, and 0.3 ml. of 50 per cent HClO₄ in the side arm of each flask. The flasks were placed on the Warburg apparatus at 38°, and measurements of oxygen uptake were made. It was found that papers in the center well were not needed to give reliable measurements of oxygen uptake or C¹⁴O₂ absorption at the rates studied. At the conclusion of the experiment, the acid in the side arm was tipped into the flask contents to denature the protein and to drive all C¹⁴O₂ into the center well. Experiments showed that complete diffusion of the CO₂ into the alkali was effected by 1 hour of further shaking. The contents of the center well were removed with an eye dropper, combined with six washings, and analyzed for radioactivity as BaC¹⁴O₃ on paper plates ((7) p. 116). The flask contents were analyzed for citrate (8) and keto acid (9) in certain instances as indicated.

Sodium acetate-1-C¹⁴ was prepared by the method of Lemmon ((7) p. 178). Cytochrome *c*, oxalacetate, α -ketoglutarate,¹ and adenosinetriphosphate were prepared in this laboratory. Other compounds were commercial products of reagent grade.

In order to prepare samples of α -ketoglutarate for determination of radioactivity an aliquot of the protein-free filtrate, containing about 3 μ M, was diluted to 3.0 ml. and mixed with 1.0 ml. of 0.1 per cent 2,4-dinitrophenylhydrazine in 2 N HCl and was allowed to stand for 20 minutes at room temperature (9). The solution was extracted with ether, and the ether extract was chromatographed on Hyflo Super-Cel (Johns-Manville), developed with ether, and eluted with water, according to the method of LePage (10). This procedure separated α -ketoglutarate from pyruvate, reagent, and other compounds. After the sample was analyzed

¹ We wish to thank Dr. W. W. Ackermann for providing this compound.

colorimetrically for α -ketoglutarate, carrier α -ketoglutarate-2,4-dinitrophenylhydrazine was added in a known amount and was then recrystallized three times from 10 per cent ethanol. The radioactivity of the product was determined following the Van Slyke oxidation and was then referred to the colorimetric value for calculation of the specific activity per micromole of α -ketoglutarate.

The acetate remaining at the completion of the experiment was determined by steam distillation of an aliquot of the acidified flask contents to which carrier sodium acetate was added, and the distillate was oxidized with persulfate. The total radioactivity in the solution or precipitate was determined by oxidation of an aliquot by persulfate, because the persulfate method gives a quantitative conversion of acetate to CO_2 , whereas acetate is incompletely oxidized by the ordinary wet and dry combustion methods.

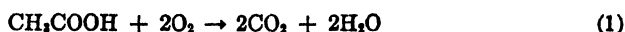
All radioactivity measurements were made on barium carbonate samples with internal counters, are corrected for self-absorption, and are expressed as counts per minute. Each sample was counted long enough to give at least 5 per cent accuracy.

Results

The fact that acetate oxidation requires (11) the presence of an additional substrate that is itself vigorously oxidized leads to difficulties in the interpretation of the results. The oxidation that is observed must be corrected for the oxidation obtainable with oxalacetate alone, and the validity of this correction might be expected to depend upon the concentration of oxalacetate. It was therefore of interest to measure not only the oxygen uptake but also the output of C^{14}O_2 when acetate-1- C^{14} was oxidized in the presence of various amounts of oxalacetate. The results of such an experiment are given in Fig. 1 which shows the oxygen uptake obtained with various levels of oxalacetate with and without $4\text{ }\mu\text{M}$ of acetate. It may be seen that the oxygen uptake is proportional to the amount of oxalacetate added when this is the sole substrate. This result is due to the fact that the system is allowed to take up oxygen until the rate has fallen to a negligible level; at this time the oxalacetate is all used up, but significant amounts of malate and citrate remain (Potter and Lyle, unpublished results).

It is apparent that the effect of the added acetate is not constant at different oxalacetate concentrations. The differences obtained by subtracting the values for the lower curve from those of the upper curve are called "extra" oxygen uptake and have been converted into micromoles of acetate at the ratio of 2 moles of O_2 per mole of acetate, since this is

the amount of oxygen required for the complete combustion of acetate according to the equation



and are plotted as "acetate oxidized" in Fig. 2. The optimal oxalacetate concentration occurred between 0.0018 and 0.0027 M. The greatest amount of extra oxygen uptake was equivalent to 95 per cent of the acetate added. In Fig. 2 are also given data on the C¹⁴O₂ as determined by the radioactivity of the center well contents. In the case of the two points nearest the optimal oxalacetate concentration, the C¹⁴O₂ amounted to approximately 60 per cent of that which would be expected on the basis of Equation 1, calculated in terms of the oxygen uptake.

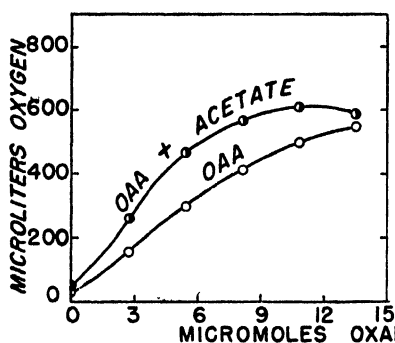


FIG. 1

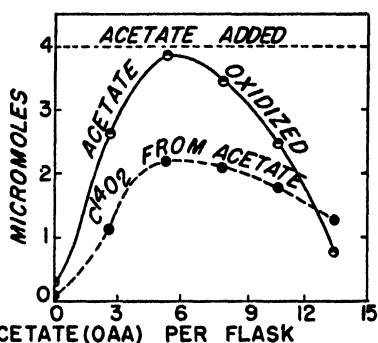
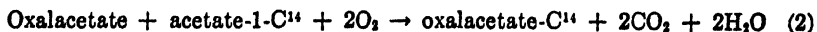


FIG. 2

FIG. 1. Oxygen uptake in 2.5 hours at varying oxalacetate concentrations in the presence and absence of 4 μM of acetate-1-C¹⁴ per flask. Kidney homogenate, 30 mg. per flask.

FIG. 2. Comparison of C¹⁴O₂ output and total acetate oxidized, as calculated from Fig. 1 and Equation 1.

The significance of the disparity between oxygen uptake and CO₂ output is that the mechanism of acetate oxidation is not represented by Equation 1 but, according to present knowledge (12), proceeds according to an over-all reaction that may be written as follows:



Thus the C¹⁴O₂ is not liberated until the labeled oxalacetate is further metabolized. No special significance can be attached to the yield of 60 per cent, since it could have been obtained in a variety of ways, depending upon the alternative pathways followed by the labeled oxalacetate and acetate and the extent of trapping in pools of the various metabolites of the Krebs cycle (see below).

Effect of Acetate-1- C^{14} Concentration on O_2 Uptake and $C^{14}O_2$ Output—Fig. 3 shows the effect of varying the acetate concentration in a system containing oxalacetate in the optimal range. There was considerable oxygen uptake in the absence of acetate, but increasing the acetate concentration produced increases in oxygen consumption up to about $10\ \mu M$ of acetate per flask, and the increase in extra oxygen uptake was paralleled by increases in $C^{14}O_2$ output. However, the yield of $C^{14}O_2$ was approximately constant at a little less than 60 per cent of the extra oxygen uptake, as in the experiment shown in Fig. 2. The extra oxygen uptake produced by 2, 4, and $10\ \mu M$ was 80, 55, and 30 per cent, respectively, of the theoretical for complete combustion of the acetate added, and the $C^{14}O_2$ output was proportional to the extra oxygen uptake.

Inhibition of $C^{14}O_2$ Output—The recovery of $C^{14}O_2$ in the above experiments was only 60 per cent, and it was considered probable that the

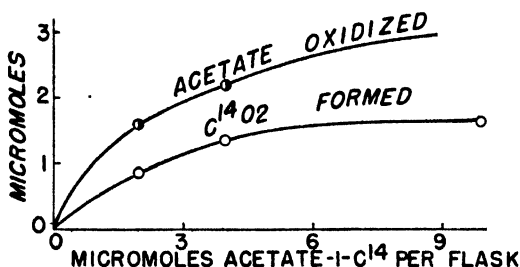


FIG. 3. Micromoles of acetate oxidized (calculated as in Fig. 2 on the basis of extra oxygen uptake) and of $C^{14}O_2$ formed in 2.5 hours by 30 mg. of kidney homogenate at various concentrations of acetate and the optimal oxalacetate concentration (see Fig. 2).

other 40 per cent was trapped in metabolic pools of Krebs' cycle intermediates, which would result from the incomplete combustion of the acetate and additional substrate. As a test of this possibility, the effect of adding pyruvate, citrate, or malonate to the reaction mixture was determined (Table I). In two experiments the standard system gave 38 and 42 per cent of the theoretical $C^{14}O_2$ and the addition of either pyruvate or citrate reduced the $C^{14}O_2$ output to 2.9 to 7.2 per cent. Malonate in low concentration almost completely suppressed the $C^{14}O_2$ output, a finding that is in harmony with Equation 2, in which no C^{14} from acetate is released as CO_2 prior to its occurrence in oxalacetate. The findings with pyruvate and citrate show that these substances can suppress $C^{14}O_2$ output and it may be inferred that the mechanism was chiefly that of diluting the C^{14} in pools of incompletely oxidized Krebs' cycle metabolites.

A more direct test of this explanation was carried out in a series of

experiments involving α -ketoglutarate, since this compound is a member of the Krebs cycle and can be very readily isolated as the 2,4-dinitrophenylhydrazone. One of several experiments involving this compound is reported in Table II, in which the standard reaction mixture was supplemented with α -ketoglutarate and the incubation time was reduced so that an appreciable amount of α -ketoglutarate remained at the end of the reaction. In addition, citrate was added in varying amounts with the idea of attempting to dilute the C¹⁴ activity of the α -ketoglutarate, since earlier negative experiments of this type with slices or minces have been cited as evidence against the participation of citrate in the Krebs cycle (12-14). It may be seen from Line 5 of Table II that the yield of C¹⁴O₂ varied from 0.9 to 2.2 per cent of the theoretical in these reaction mixtures in which α -ketoglutarate was added, in contrast to yields of 37 to 60 per cent in experiments with the compound absent. The α -keto-

TABLE I

Effect of Various Additions on C¹⁴O₂ Output and O₂ Uptake

4 μ M of labeled acetate were added to each flask and incubated 150 minutes with 0.3 ml. of 10 per cent kidney homogenate in the standard reaction mixture.

Addition to standard mixture	Per cent original C ¹⁴ found in center well		O ₂ uptake (10-150 min.)	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
			μ l.	μ l.
None	38.0	42.0	370	480
0.005 M pyruvate.	4.3	2.9	230	190
0.005 " citrate	5.3	7.2	370	570
0.004 " malonate	0.9	1.2	140	143

glutarate remaining at the end of the incubation period contained considerable radioactivity (Lines 8 and 9) and this amounted to 25 to 38 per cent of the activity of the acetate that had disappeared (Line 11). The addition of citrate to the reaction mixture resulted in a lowering of the specific activity of the α -ketoglutarate (Line 8), as well as a lowering of the total activity of the α -ketoglutarate per mole of utilized acetate (Line 11). These experiments show that C¹⁴ from acetate can be incorporated into α -ketoglutarate and that, contrary to the previous experiments (with different tissue preparations), the resulting activity is diluted by the addition of citrate. The pathways of acetate metabolism other than that of the Krebs cycle are probably of no quantitative significance in these experiments.² In one experiment of 2.5 hours duration

² Bloch and Kramer (15) have reported that 0.3 per cent of the added acetate was incorporated into fatty acids in liver slices.

with kidney homogenate the precipitated protein was centrifuged, washed five times with 2 ml. portions of 5 per cent HClO_4 , and assayed for C^{14} . Of the original activity added as acetate, 63 per cent was in the protein-free supernatant fluid, 37.5 per cent was in the center well, and none was in the protein precipitate. Analysis of the supernatant fluid showed that acetate equivalent to 10 per cent of the original amount was still present. In a similar experiment with approximately 30 mg. of slices of Flexner-Jobling carcinoma, 87 per cent of 4 μM of C^{14} -acetate remained

TABLE II

Formation of α -Ketoglutarate from Acetate in Kidney Homogenates

30 μM of α -ketoglutarate added. Specific activity of acetate 125,000 c.p.m. per μM . 1 hour's incubation.

Line No.					
1	Citrate added, μM	0.0	5.0	10.0	15.0
2	" final, "	0.5	2.0	4.4	8.2
3	Acetate- C^{14} added, μM	1.7	1.7	1.7	1.7
4	" final, "	0.17	0.19	0.26	0.43
5	C^{14} in center well, %	2.2	1.5	1.1	0.9
6	O_2 uptake (10 min. to end), μl	325	306	324	332
7	α -Ketoglutarate final (colorimetric), μM	20	28	29	31
8	Specific activity of α -ketoglutarate, c.p.m. per μM	3,150	2,550	1,740	1,280
9	Total activity of α -ketoglutarate, c.p.m.	69,400	71,500	51,000	39,400
10	Final α -ketoglutarate from C^{14} -acetate, μM	0.56	0.58	0.41	0.32
11	α -Ketoglutarate from C^{14} -acetate, μM	0.37	0.38	0.28	0.25
	C^{14} -Acetate utilized				

unchanged after 2.5 hours, and 4.0 per cent of the activity was found in the center well. In separate experiments with kidney and tumor homogenates no radioactivity was obtained when the steam-volatile material was made slightly alkaline and redistilled as a test for acetoacetate.

Oxygen Uptake and C^{14}O_2 Output by Various Tissues—In Table III are given the results of studies involving both slices and homogenates of various tissues. The last column shows the per cent conversion of C^{14} -acetate to C^{14}O_2 . In the case of kidney, the slices converted essentially all of the acetate to CO_2 , while the homogenates yielded only 38 and 52 per cent of the theoretical C^{14}O_2 in the two experiments reported here, in which the results are similar to data reported earlier.

TABLE III

CO₂ Production from Acetate by Various Rat Tissues

The incubation time was 150 minutes. The specific activity of the acetate was 1050 c.p.m. per μ l. The figure in the last column was obtained by dividing the counts found in the center well by the specific activity of the acetate.

Tissue	Wet weight per flask*	Acetate added	Initial rate of oxygen uptake	O ₂ in 150 min.	Total counts per min. in center well	Conversion of acetate to CO ₂
Homogenates						
	mg.	μ M	μ l. per 10 min.	μ l.		per cent
Kidney	30	4	75	460	2,200	52
"	30	4	65	370	1,610	38
Liver...	60	4	48	540	1,320	31
"	60	4	55	550	1,650	37
Heart....	60	4	63	290	680	16
"	50	10	44	201	740	7.0
Brain....	80	4	58	180	68	1.6
"	80	4	44	180	64	1.5
Muscle	80	4	52	117	25	0.60
"	60	10	42	180	57	0.54
Slices						
Kidney	47	10	84	470	10,100	96
"	35	4	51	590	3,920	92
Liver..	58	10	32	370	1,680	16
"	50	10	33	380	2,570	25
Lung.	32	4	17	195	680	16
"	12	4	7	69	285	6.7
Flexner-Jobling carcinoma	30	4	17	215	184	4.1
"	44	10	20	263	103	0.98
"	28	4	13	125	140	3.3
Walker carcinosarcoma No. 256.	22	10	17	196	152	1.5
Walker carcinosarcoma No. 256.	26	4	20	254	174	4.1

* In the slice experiments the weight given was the final wet weight of the blotted slices after treatment in the flask with HClO₄. Experiments showed that the initial dry weight was 0.48 of this value for both kidney and the Walker carcinosarcoma No. 256.

The production of C¹⁴O₂ from labeled acetate in homogenates parallels the ability to remove oxalacetate and pyruvate (Potter and Lyle, unpublished results). As with muscle, the output of C¹⁴O₂ by homogenates of thymus, spleen, lung, Flexner-Jobling carcinoma, and Walker carcino-

sarcoma No. 256 was always considerably less than 1 per cent and is not reported in Table III. Table III shows that, in contrast to the complete conversion of acetate to CO_2 in the kidney slices, the rat tumor slices were extremely low in activity. The kidney slice must have been capable of converting acetate to CO_2 at a higher rate than was actually observed, since in the time observed the conversion was complete. The tumor slices contained not more than one-twenty-fifth of the activity demonstrated by kidney and thus the actual ability of the tumor slice to oxidize acetate via the Krebs cycle must be very small and well below the magnitude that would be significant in oxygen uptake measurements.

DISCUSSION

The present studies supplement previous indications (11, 14, 16) that acetate is oxidized via the Krebs cycle. The necessity for adding oxalacetate as a cooxidant (Fig. 1), the conversion of acetate into α -ketoglutarate (Table II), the effect of citrate upon the latter reaction (Table II), the suppression of C^{14}O_2 output by α -ketoglutarate, citrate, pyruvate, and malonate (Tables I and II), all constitute evidence in favor of participation in the Krebs cycle.

The suppression of C^{14}O_2 production from acetate in the presence of malonate (Table I) is of particular interest in connection with the Krebs cycle because, with the present evidence that citrate is the product of the condensation between acetate and oxalacetate (16), it might have been assumed that there would be a 50 per cent conversion of the C^{14} to C^{14}O_2 in the transformation of citrate to succinate in the presence of malonate, since citrate is a symmetrical molecule. However, it has been shown in this laboratory that the citrate molecule is metabolized asymmetrically (17), and therefore the label in the citrate molecule that is produced from the isotopic acetate must be present in the succinate and not in the CO_2 eliminated in the conversion of α -ketoglutarate to succinate. The low yield of C^{14}O_2 in a system in which the cycle has been blocked by malonate is thus further evidence in favor of the asymmetrical nature of C^{14} -labeled citrate in this system.

Oxygen uptake has been used by numerous investigators prior to the advent of isotopically labeled acetate as a measure of the oxidation. However, the measurement of oxygen uptake is clearly inadequate as a measure of oxidation, as has been shown in Figs. 1 to 3, because of the necessity of adding another substrate, which itself is oxidized and may form "active" acetate preferentially. This conclusion applies to slices as well because of the oxidation of endogenous substrates. The measurement of the C^{14}O_2 output from labeled acetate is a more direct measure of acetate oxidation than is the oxygen uptake, but interpretation is made

difficult because of dilution of the radioactivity by pools of unlabeled substrates, as has been shown in Tables I and II. These pools may be larger in the homogenate than in the slice since it is necessary to add appreciable amounts of substrate in order to maintain enzymatic activity (3, 18). In the case of the slice, although there are some pools of endogenous substrates, they probably do not dilute the substrate to as great an extent, and the activity is maintained over a longer period. These considerations are believed to account for the fact that kidney slices converted 92 to 96 per cent of the added acetate to C¹⁴O₂, whereas the kidney homogenates converted only 37 to 60 per cent to C¹⁴O₂ before they became inactive. In spite of these possible uncertainties, our experiments strongly suggest that in tumor and in some normal tissue slices the rate of acetate oxidation must be small in terms of Q_{O₂} and support earlier observations on tumor slices by Elliott *et al.* (2), as well as the data on homogenates (3, 4) and recent studies on citrate production *in vivo* (19). All of these studies support the view that the Krebs condensation or a closely related reaction takes place only to a limited extent in tumor tissue, or that tumor tissues are less able to activate acetate than are various other tissues.

SUMMARY

1. A study of the oxidation of acetate-1-C¹⁴ by slices and by homogenates of rat tissues has been carried out.

2. Acetate was oxidized in the system that was designed for the oxidation of oxalacetate by homogenates provided that oxalacetate was also present. Acetate alone was not acted upon, and all of the results indicate that acetate must condense with oxalacetate to form citrate, which is then oxidized via the Krebs cycle. The addition of pyruvate, citrate, α -ketoglutarate, or malonate suppressed the output of C¹⁴O₂, and it was shown that C¹⁴ was incorporated into α -ketoglutarate. Added citrate decreased the extent of the latter reaction.

3. In the case of kidney slices essentially all of the labeled acetate was converted to C¹⁴O₂, but with kidney homogenates the conversion did not exceed 60 per cent. It was concluded that in the latter instance the remaining activity was trapped in metabolites that remained at the time the enzymes in the homogenate became inactive.

4. A number of normal tissues and two transplantable rat tumors were studied in the form of both slices and homogenates and both oxygen uptake and C¹⁴O₂ output were measured. The tumors exhibited less than one-twenty-fifth of the activity of the kidney tissue, and other tissues fell between these two extremes.

5. The C¹⁴O₂ output of slices in the presence of labeled acetate was considered to be the best available criterion of acetate oxidation but a number of limitations of both homogenates and slices were pointed out.

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CONVERSION OF MONOMETHYL-L-TRYPTOPHAN TO NIACIN AND *N*¹-METHYLNICOTINAMIDE BY THE RAT

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Gordon and Jackson (1) found that synthetic monomethyltryptophan was capable of stimulating growth in rats subsisting on a diet deficient in tryptophan. Chin (2) isolated kynurenine and kynurenic acid from the urine of rabbits after the parenteral administration of monomethyl-L-tryptophan (MMT).

On the other hand, recent studies have shown that the albino rat, pig, horse, cotton-rat, and man can convert dietary tryptophan to niacin or its methylated derivative, *N*¹-methylnicotinamide (*N*¹-Me) (3).

In the present paper the urinary excretion of niacin and *N*¹-Me by rats after the ingestion of MMT was studied.

EXPERIMENTAL

The MMT employed in this study was isolated from the seeds of *Abrus precatorius*, which grows wild in the south of Formosa. The compound was thrice recrystallized from hot water, m.p. 296° (with decomposition), and contained 12.72 per cent of nitrogen.

Male white rats were used as experimental animals. For several weeks they were fed a definite amount of basal diet with the following per cent composition: soy bean flour 75, bran 20, and salt 5. In addition, each received daily 0.1 ml. of cod liver oil. The animals were divided into three groups. Six animals, weighing between 112 and 167 gm., were used in Groups 1 and 2. Five weanling rats, weighing between 30 and 40 gm., were designated as Group 3. They were placed in metabolism cages and urine collections were made at 9.30 a.m. every day from each of the three groups (not from the individual animals). The 24 hour urine specimens were preserved with 3 ml. of concentrated hydrochloric acid. In both Groups 1 and 2, 100 mg. of MMT were then fed per rat per day for 3 days. In Group 3 each rat received 50 mg. of MMT per day. MMT was mixed thoroughly with a definite amount of cooked rice, which was administered throughout the experimental period. MMT supplements

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TABLE I

Influence of Administration of MMT on Urinary Excretion of Niacin and N¹-Me† by Rats*

Day	Dietary regimen	Group 1 (3 rats)		Group 2 (3 rats)		Group 3 (5 rats)	
		Niacin	N ¹ -Me	Niacin	N ¹ -Me	Niacin	N ¹ -Me
1	Basal	73.5	680.6	78.0	840.0	19.2	191.0
2	"	39.6	399.9	91.8	864.9	17.0	252.9
3	"	62.4	430.0	55.6	755.4	33.2	260.4
4	"	75.2	432.0	49.6	720.6	24.3	197.9
5	" + MMT‡	191.4	959.6		1879.6	80.5	508.9
6	" + "	173.8	1140.0	145.3	2390.3	120.0	1041.5
7	" + "	305.1	1233.9		2282.2	153.3	1260.0
8	"	151.2	1120.0		1127.8	209.1	536.7
9	"	56.1	906.2		874.2	151.8	374.4
10	"	65.1	722.3		519.8	32.3	675.2
11	"	26.0	633.6		608.8	67.5	689.5

* 1 galvanometer division of the Fisher electrophotometer was taken as the unit of the amount of niacin excreted in 24 hours by all the rats of the group.

† The amount of N¹-Me excreted in 24 hours by all the rats of the group, which is equivalent to the fluorescence of 1 γ of quinine sulfate.

‡ To Groups 1 and 2, 100 mg. of MMT per rat per day were administered, but to Group 3, 50 mg. of MMT per rat per day.

TABLE II

Influence of Administration of Tryptophan on Urinary Excretion of Niacin and N¹-Me by Rats (Group 4)

Day	Dietary regimen	Niacin*	N ¹ -Me*
1	Basal	133.4	405.5
2	"	117.6	499.3
3	"	192.5	700.0
4	"	154.3	465.9
5	" + tryptophan†	72.0	895.2
6	" + "	285.6	1087.8
7	" + "	234.9	1827.9
8	" + "	416.5	1937.2
9	" + "	262.6	2179.8
10	"	105.4	810.5
11	"	172.5	477.0
12	"	118.8	564.4
13	"	121.3	629.1

* See Table I for an explanation of these units.

† 93.5 mg. of L-tryptophan per rat per day were administered for 5 days.

were given at 9.30 a.m. The animals were hungry at that time and ate the supplements almost completely (Table I).

Niacin was determined colorimetrically (4). N^1 -Me was determined fluorometrically (5). These determinations were made on the pooled urine from each group of the rats (not on the urine from individual animals).

To compare the effect of MMT and L-tryptophan upon the excretion of niacin and N^1 -Me, 93.5 mg. of L-tryptophan per rat per day were administered for 5 days to another group of three rats (Group 4, 165 to 183 gm. in weight) kept under the same experimental conditions as Groups 1, 2, and 3 (see Table II).

The results are presented in Tables I and II. Since neither niacin nor N^1 -Me was available in our laboratory, 1 galvanometer division of the Fisher electrophotometer was used as an arbitrary unit for the amount of

TABLE III
Influence of Parenteral Administration (Subcutaneous) of MMT on Urinary Excretion of Niacin and N^1 -Me by Rats

Day	Dietary regimen	Niacin*	N^1 -Me*
1	Basal	102.0	771.0
2	"	126.0	896.0
3	"	171.0	895.8
4	"	81.0	540.0
5	" + MMT†	187.0	1060.8
6	" + "	355.5	1935.0
7	" + "	382.2	1986.4
8	"	111.6	1463.0
9	"	112.2	748.0
10	"	195.3	693.0
11	"	207.0	683.1

* See Table I for an explanation of these units.

† 100 mg. of MMT per rat per day were injected.

niacin. The same method was used for the estimation of N^1 -Me and is equivalent to the fluorescence of 1 γ of quinine sulfate.

RESULTS AND DISCUSSION

The data presented in Table I demonstrate the conversion of MMT to niacin and N^1 -Me. Although it is difficult to estimate from the data presented here how much MMT was converted to niacin and N^1 -Me, it is evident that the rat can convert dietary MMT to niacin derivatives.

The average daily niacin excretion of the three groups on the basal diet was 62.7, 68.8, and 23.4 units respectively (Table I). The addition of MMT to the basal diet led to a prompt increase in the excretion of niacin, the maximum values being 305.1, 145.3, and 209.1 units. After the supplement was stopped, the increased excretion persisted for 2 or

3 days before returning to normal. Similar results were observed in N^1 -Me excretion. Animals on the basal diet excreted an average of 485.6, 801.2, and 225.6 units of N^1 -Me in 24 hours. Increased excretion of N^1 -Me was caused by the administration of MMT. The maximum excretion was 1233.9, 2390.3, and 1260.0 units respectively. The N^1 -Me excretion of animals on the basal diet did not return immediately to normal after the MMT supplement was stopped.

The results presented in Table II show that there are no considerable differences between the behavior of MMT and that of L-tryptophan.

Ling and Tung (6) have suggested that the production of formaldehyde and amino acids by oxidative demethylation of monomethyl-L-amino acids by demethylase appears to be a general reaction. It is possible that MMT is demethylated and metabolized as tryptophan. The results of our study confirm this idea.

Since the demethylase content in the liver and kidney of rats is negligible within experimental error (7), and since Wang and Tung (unpublished), investigating the distribution of demethylase in bacteria, found that the demethylase content of *Escherichia coli* was considerable, it seemed to us that MMT might be demethylated by the intestinal microorganisms. To test this, we injected 100 mg. of MMT subcutaneously per rat per day into another group of rats under the experimental conditions described above. The results presented in Table III show that the demethylation and conversion of MMT to niacin derivatives occur in the rat even after parenteral administration.

SUMMARY

1. The conversion of monomethyl-L-tryptophan (MMT) to niacin and N^1 -methylnicotinamide has been studied in the rat.
2. When rats were fed MMT in addition to the basal diet, a marked increase in the urinary excretion of niacin derivatives was observed.
3. The demethylation and conversion of MMT to niacin derivatives occur in the rat even after parenteral administration.

We wish to express our appreciation to Dr. Philip P. Cohen and Dr. Charles Heidelberger for valuable suggestions.

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THE NON-UTILIZATION OF ACETYLDEHYDROAMINO ACIDS FOR GROWTH BY THE RAT*

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The utilization of dehydroamino acids by the animal body has been postulated as a phase of intermediary nitrogen metabolism (1-5). Hoberman and Fruton (6) have shown that the nitrogen of acetyldehydrotyrosine is not utilized by the fasting rat. With the exception of acetyldehydroalanine, dehydropeptidases isolated from rat liver and kidney have been shown by Price and Greenstein (7) not to hydrolyze acetyldehydroamino acids. Cooley and Wood (5) found that the intact animal did not utilize acetyldehydrotryptophan for growth.

In order to extend the generality of the concept that acetyldehydro compounds cannot be utilized, acetyldehydro derivatives of two additional essential amino acids have been studied with young rats. Acetyldehydrophenylalanine was chosen as a derivative of an essential amino acid which is utilizable in both the D and L forms. Acetyldehydrovaline represented amino acids of which only the L form supports growth of the rat. The acetyldehydro and acetyl derivatives and the keto acid analogues of each amino acid were prepared and fed diets deficient in the corresponding amino acid. Neither acetyldehydrophenylalanine nor acetyldehydrovaline supported growth. Similar results with acetyldehydrophenylalanine have recently been reported by Armstrong and Lewis (8). The diets were adequate for growth when supplemented with either the amino acid, its acetyl derivative, or the corresponding keto acid. The latter results confirm previous reports (9-12).

EXPERIMENTAL^{1, 2}

Preparation of Compounds—Acetyldehydrophenylalanine was prepared from acetylglycine by the method of Herbst and Shemin (13). The melting point of the preparations used was 191°. Acetyl-DL-phenylalanine,

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¹ The authors wish to thank Merck and Company, Inc., the Huron Milling Company, and The Dow Chemical Company for supplies of amino acids used in the diets.

² All melting points are corrected.

TABLE I

Growth Response to Phenylalanine, Acetyl-DL-phenylalanine, Acetyldehydrophenylalanine, and Sodium Phenyl Pyruvate

Initial weight of rat and sex	No. of days	Total weight of food eaten	Change in weight	Supplement*
gm.		gm.	gm. per day	
81 ♂	4	33	+1.5	DL-Phenylalanine
	4	17	-2.7	None
	20	94	-0.6	Acetyldehydrophenylalanine (1)
69 ♂	4	18	+2.2	DL-Phenylalanine
	4	33	+1.7	"
	4	15	-2.1	None
	8	34	-0.5	Acetyldehydrophenylalanine (1)
81 ♂	12	52	-0.2	" (2)
	4	19	+2.0	DL-Phenylalanine
	4	32	+1.0	"
	4	23	-2.8	None
64 ♂	8	43	-0.4	Acetyldehydrophenylalanine (1)
	16	65	-0.5	" (2)
	4	29	+1.4	DL-Phenylalanine
	4	15	-2.1	None
74 ♀	8	42	+0.8	Sodium phenyl pyruvate (1)
	12	72	+0.9	" " " (2)
	4	17	+0.8	DL-Phenylalanine
	4	31	+1.7	"
73 ♂	4	15	-2.2	None
	8	48	+1.0	Sodium phenyl pyruvate (1)
	12	86	+1.6	" " " (2)
	4	18	+1.0	DL-Phenylalanine
71 ♂	4	30	+1.7	"
	4	17	-2.5	None
	8	40	+0.4	Acetyl-DL-phenylalanine (1)
	12	70	+1.4	" (2)
71 ♂	4	22	+1.2	DL-Phenylalanine
	4	29	+1.4	"
	4	17	-2.5	None
	8	44	+1.0	Acetyl-DL-phenylalanine (1)
	12	73	+1.3	" (2)
	4	20	+0.8	DL-Phenylalanine

* DL-Phenylalanine was added to the basal diet at a level of 1.37 per cent. The numbers in the parentheses indicate the molar equivalent to DL-phenylalanine at which the derivatives were added.

m.p. 151°, was obtained by catalytic hydrogenation of acetyldehydrophenylalanine over platinum oxide. Phenylpyruvic acid, m.p. 152°, was prepared by acid hydrolysis of acetyldehydrophenylalanine. Acetyldehydrovaline, m.p. 200°, was made by heating chloroacetyl-DL-valine (14)

TABLE II
*Growth Response to Valine, Acetyl-DL-valine, Acetyldehydrovaline, and
 Sodium Dimethyl Pyruvate

Initial weight of rat and sex	No. of days	Total weight of food eaten	Change in weight	Supplement*
gm.		gm.	gm. per day	
74 ♂	8	53	+1.8	DL-Valine
	4	29	-2.8	None
	16	108	+2.3	Sodium dimethyl pyruvate (1)
68 ♂	4	22	+0.7	DL-Valine
	4	27	-2.5	None
	16	118	+2.2	Sodium dimethyl pyruvate (1)
	8	38	-2.8	Acetyldehydrovaline (2)
62 ♀	4	25	+1.0	DL-Valine
	4	29	-2.2	None
	4	25	+2.6	Sodium dimethyl pyruvate (1)
	12	77	+1.1	" " " (4)
	8	36	-2.5	Acetyldehydrovaline (2)
56 ♀	8	44	+0.9	DL-Valine
	4	27	-2.9	None
	8	43	-0.8	Acetyldehydrovaline (1)
	8	36	-0.3	" (2)
58 ♀	8	46	+1.1	DL-Valine
	4	29	-1.2	None
	8	38	-1.2	Acetyldehydrovaline (1)
	8	40	-0.4	" (2)
67 ♀	8	60	+2.1	DL-Valine
	12	100	+0.5	Sodium dimethyl pyruvate (4)
	8	31	-1.2	Acetyldehydrovaline (2)
64 ♂	28	200	+1.2	DL-Valine
55 ♂	12	54	-1.2	None
	12	74	+1.7	Acetyl-DL-valine
56 ♂	12	52	-1.5	None
	16	112	+1.1	Acetyl-DL-valine
56 ♀	12	50	-1.0	None
	8†	28	-0.6	Acetyldehydrovaline (2)
53 ♀	12†	53	-1.2	None
55 ♂	12†	52	-1.3	"

* DL-Valine was added to the basal diet at a level of 2.28 per cent. The numbers in the parentheses indicate the molar equivalent to DL-valine at which the derivatives were added.

† The animals died at this time.

with acetic anhydride according to the procedure of Bergmann and Stern (2). Dimethylpyruvic acid was obtained by acid hydrolysis of 2-phenyl-4-isopropylidene-5-oxazolone (15). The boiling point of the keto acid was 65° at 15 mm. of Hg pressure. Acetyl-DL-valine, m.p. 146°, was

prepared by acetylation of DL-valine with acetic anhydride (16). In all instances the α -keto acids were converted to the more stable sodium salts.

Feeding Experiments—Young rats, litter mates of the Yale strain, were used for assaying the growth-supporting properties of the derivatives. The animals were kept in individual cages and given food and water *ad libitum*. A mixture of crystalline amino acids which has been shown by Rose, Oesterling, and Womack (17) to support growth was used as the source of amino nitrogen in the diet. The composition of the mixture was L-leucine 1.37, DL-isoleucine 1.82, DL-methionine 0.91, DL-phenylalanine 1.37, DL-threonine 1.60, DL-tryptophan 0.46, L-glutamic acid 2.00, L-lysine hydrochloride 1.71, L-histidine hydrochloride 1.08, L-arginine hydrochloride 0.56, DL-valine 2.28, sodium bicarbonate 1.44. In each series the amino acid corresponding to the derivative under test was omitted from the mixture and the derivative was added at molar equivalent levels. The composition of the basal diet was amino acids 16.60, sucrose 15.00, dextrin 59.65, Cellu flour 2.00, salt mixture, Jones and Foster (18), 4.00, corn oil 2.00, cod liver oil 0.05, inositol 0.10, choline chloride 0.20, liver extract, Wilson's 1:20 liver powder, 0.40. Water-soluble vitamins were added at the time of feeding. The following supplements were added per kilo of diet: thiamine hydrochloride 5 mg., riboflavin 10 mg., pyridoxine hydrochloride 5 mg., nicotinic acid 5 mg., calcium *d*-pantothenate 25 mg., *p*-aminobenzoic acid 300 mg., 2-methyl-1,4-naphthoquinone 2 mg. Weighings were made every 4 days. Growth measured as a change in weight is shown in Tables I and II.

SUMMARY

When acetyldehydrophenylalanine was fed with a phenylalanine-deficient diet, it did not support the growth of young rats. Likewise, acetyldehydrovaline did not serve in the place of valine in the diet of the rat. In confirmation of earlier investigations, sodium phenyl pyruvate or acetyl-DL-phenylalanine supported growth in the absence of phenylalanine; sodium dimethyl pyruvate or acetyl-DL-valine replaced valine.

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THE NATURE OF THE TJ FACTOR FOR *LACTOBACILLUS LACTIS* DORNER

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Two unidentified growth factors have been reported for *Lactobacillus lactis* Dorner. One of these is a component of liver extract, designated LLD, and the other, TJ, is found in tomato juice (1, 2). When vitamin B₁₂ became available as a chemically pure source of LLD activity (3, 4), preliminary studies directed toward the isolation of the TJ principle were undertaken. Earlier work from this laboratory has shown that in a D-alanine medium the added growth response elicited by a tomato juice eluate could be replaced by a mixture of fumaric acid and sodium ethyl oxalacetate (5). In testing a variety of medium constituents, Shorb (6) subsequently showed that D-alanine and tomato juice are interchangeable in a medium containing sodium ethyl oxalacetate.¹ While L-alanine is devoid of TJ activity, it is essential for growth of the microorganism.

A more detailed investigation of the D-alanine-acid relationship indicated that D-alanine was essential for growth, while the acids allowed an additional growth response. Although D-alanine alone will support three-quarters maximal growth, no growth was obtained with the acids in the absence of D-alanine.

Since it has been demonstrated that for certain lactobacilli one of the functions of vitamin B₆ is concerned with the synthesis of D-alanine (7), it appeared likely that the activity of tomato juice eluate in replacing D-alanine might be related to the B₆ vitamins. In this respect pyridoxine and pyridoxamine, at levels adequate for other lactic acid bacteria (2.0 and 0.4 γ per ml. respectively), were inactive. The observation that pyridoxal phosphate and pyridoxamine phosphate are more readily utilized by several lactic acid bacteria (8) than the non-phosphorylated forms suggested that the TJ factor might be related to these compounds.

This paper describes observations made on the relationship of the TJ factor and the B₆ vitamins to the growth of *L. lactis* Dorner and to certain other lactobacilli.

EXPERIMENTAL

Medium—The basal medium used in the studies with *L. lactis* was a modification of the medium previously described for the microbiological

¹ Personal communication from Dr. M. S. Shorb, Department of Poultry Husbandry, University of Maryland.

estimation of LLD activity (5).² When the ATCC strain 8000 was used, the medium was further supplemented with Tween 80 (0.05 mg. per ml.). The medium described by McNutt and Snell³ was used for *Lactobacillus acidophilus* and *Lactobacillus helveticus*.

Inoculum—Stock cultures of *L. lactis* ATCC 10697 and ATCC 8000 were maintained and the inoculum was prepared as previously described (5). *L. helveticus* and *L. acidophilus*⁴ were maintained in semisolid milk agar. Inoculum was prepared from a 24 hour broth culture grown in a basal medium supplemented with pyridoxal phosphate 0.1 γ per ml., yeast extract 0.5 per cent, and Bacto-peptone 0.5 per cent.⁵

Procedure—Solutions to be tested were added to lipless (180 \times 22 mm.) test-tubes. Distilled water was added to bring the volume to 5.0 ml., followed by 5.0 ml. of double strength basal medium. The tubes were plugged with cotton and sterilized at 15 pounds pressure for 13 minutes. Each tube was inoculated with 1 drop (0.05 ml.) of a culture suspension (5). The tubes were then incubated at 37° for 40 hours in the case of *L. lactis* and growth was determined by titrating the lactic acid formed with 0.1 N sodium hydroxide. In the case of *L. helveticus* and *L. acidophilus* growth was determined turbidimetrically after 24 hours incubation at 37° with the Evelyn photometer fitted with a 540 m μ filter.

RESULTS AND DISCUSSION

Half maximal growth values obtained with the four test organisms show that pyridoxal phosphate and pyridoxamine phosphate are the most active of the B₆ vitamins (Table I). Pyridoxamine phosphate was the most active for all three organisms, being 3 to 7 times more active than pyridoxal phosphate. A similar ratio was reported with *L. helveticus* and *L. acidophilus* (8). Because of the relatively high concentrations of pyridoxine, pyridoxal, pyridoxamine, or DL-alanine required for half maximal growth, their interference in the assay for phosphorylated forms of vitamin B₆ is negligible in the assay of natural materials. The possible inter-

² This medium was modified by eliminating pyridoxamine, fumaric acid, and sodium ethyl oxalacetate, replacing DL-alanine with L-alanine, supplementing the basal medium with 0.2 m μ gm. per ml. of vitamin B₁₂, and using a total volume of 10 ml.

³ The composition was obtained from Dr. E. E. Snell in a private communication. A modification of this medium was recently published by McNutt and Snell (9). The medium used differs from the one published as follows: NaCl 0 mg., MnSO₄·H₂O 45 mg., calcium pantothenate 2 γ , *p*-aminobenzoic acid 1 γ , niacin 10 γ per 5 ml. of double strength medium. Oleic acid was eliminated since it was found unnecessary when Tween 80 was used.

⁴ Cultures obtained from E. E. Snell, Department of Biochemistry, University of Wisconsin.

⁵ Dr. E. E. Snell, personal communication.

ference of pyridoxal when *L. lactis* is used is minimized by converting it to pyridoxamine through autoclaving the samples in the medium.

Two TJ preparations of different potencies were evaluated for pyridoxal phosphate and pyridoxamine phosphate content with the three test species. The estimation of pyridoxal phosphate was based on the quantitative conversion to pyridoxamine phosphate by autoclaving in the presence of glutamic acid (10). TJ preparations were therefore tested under three conditions: Procedure A, aseptic addition to medium after sterilization by filtration through an ultrafine sintered glass filter, Procedure B, autoclaving with medium, and Procedure C, autoclaving with glutamic acid followed by Procedure B. The results thus obtained are presented in Table

TABLE I
Response of Lactic Acid Bacteria to DL-Alanine and Vitamin B₆ Compounds

Compound	Requirement for half maximal growth, γ per ml.			
	<i>L. lactis</i> Dorner		<i>L. helveticus</i>	<i>L. acidophilus</i>
	ATCC 10697	ATCC 8000		
DL-Alanine.....	73	33	7.0	22
Pyridoxine·HCl.....	40	40	>400	>400
Pyridoxamine.....	1.1	1.1	20	22.5
Pyridoxal*.....	0.09	0.1	>100	14
Pyridoxal phosphate*.....	0.0053	0.0020	0.0006	0.0003
Pyridoxamine phosphate*†.....	0.0002	0.0003	0.0001	0.0001

* Added aseptically after sterilization by filtration through an ultrafine sintered glass filter. We are indebted to Dr. S. A. Harris and Mr. A. N. Wilson for synthetic calcium pyridoxal phosphate, estimated to be 75 per cent pure. The above values are corrected on this basis.

† Pyridoxamine phosphate was prepared from pyridoxal phosphate by the procedure of Rabinowitz and Snell (10). The conversion was assumed to be quantitative.

II. With both samples and with all test organisms 1 TJ unit⁶ was found to be equivalent to approximately 2 μ gm. of pyridoxamine phosphate under conditions of the TJ assay (Procedure B). No significant difference in the pyridoxamine phosphate equivalence of the concentrate was observed when it was assayed by the three procedures, indicating that its TJ activity is primarily due to pyridoxamine phosphate or a pyridoxamine phosphate-like substance. However, Wilson's liver paste (70 per cent ethanol-soluble fraction), which was low in TJ activity, showed an approximately 7-fold increase in activity with *L. lactis* and a 3-fold increase

⁶ 1 TJ unit is equivalent to that amount of TJ per 10 ml. required for half maximal growth of *L. lactis*.

with *L. helveticus* and *L. acidophilus* when the sample was autoclaved with the medium. A similar ratio was found with these organisms for pyridoxal phosphate and pyridoxamine phosphate (Table I). These data indicate that the TJ activity of Wilson's liver paste is primarily due to pyridoxal phosphate.

No consistent differences were noted in values for pyridoxamine phosphate between samples autoclaved with the medium or treated with glutamate prior to assay, indicating that a quantitative conversion of pyridoxal phosphate to pyridoxamine phosphate is effected by autoclaving in the amino acid medium.

TABLE II

*Pyridoxamine Phosphate Equivalence of TJ Preparations (μgm. Per TJ Unit)**

Source	TJ activity	<i>L. lactis</i> Dorner ATCC 8000			<i>L. helveticus</i>			<i>L. acidophilus</i>		
		Pro- cedure A†	Pro- cedure B‡	Pro- cedure C§	Pro- cedure A†	Pro- cedure B‡	Pro- cedure C§	Pro- cedure A†	Pro- cedure B‡	Pro- cedure C§
	units per mg.†									
Wilson's liver paste.....	16	0.2	1.5	2.3	0.5	1.5	2.3	0.5	2.3	1.5
<i>S. griseus</i> concentrate....	3000	1.5	1.5	1.5	1.5	0.8	0.8	1.5	2.3	2.3

* The pyridoxamine phosphate values have been corrected as in Table I.

† Aseptic addition of sample after sterilization by filtration through ultrafine sintered glass filter.

‡ Sample autoclaved with medium.

§ Sample autoclaved with glutamic acid prior to autoclaving with medium.

¶ 1 TJ unit is equivalent to that amount of TJ required for half maximal growth of *L. lactis*.

The data in Table II indicate that the TJ activity of the *Streptomyces griseus* concentrate was due to pyridoxamine phosphate. To investigate further this relationship, a comparison was made of the chemical properties of the active constituent of the concentrate and of synthetic pyridoxamine phosphate.⁷ Pyridoxamine phosphate (3 γ per ml.), pyridoxal phosphate (3 γ per ml.), and the concentrate (1 mg. per ml.) were autoclaved in 0.055 N hydrochloric acid at 20 pounds pressure (10). Aliquots were withdrawn at intervals and assayed by Procedure B. The results are illustrated in Fig. 1. Under these conditions, the active constituent of the concentrate could not be distinguished from pyridox-

⁷ The pyridoxamine phosphate used in these experiments was a synthetic product estimated to be about 25 per cent pure, for which we are indebted to Dr. D. A. Heyl and Miss Eileen Johnston.

amine phosphate, whereas pyridoxal phosphate is much more rapidly hydrolyzed. Furthermore, both the concentrate and pyridoxamine phosphate were inactivated upon contact for 90 minutes at room temperature with 1 per cent sodium nitrite made 0.01 N with respect to hydrochloric acid (11). Both pyridoxamine phosphate and the active fraction of the concentrate were adsorbed on the cation exchanger IR-120 in the hydrogen cycle and eluted with aqueous ammonium hydroxide. The same two preparations, both separately and in admixture, were adsorbed quantitatively on activated alumina from 90 per cent methanol solution; washing with 50 per cent methanol containing 5 to 10 per cent of ammonium hydroxide failed to remove microbiologically active material. Recovery was accomplished by elution with aqueous ammonium hydroxide.

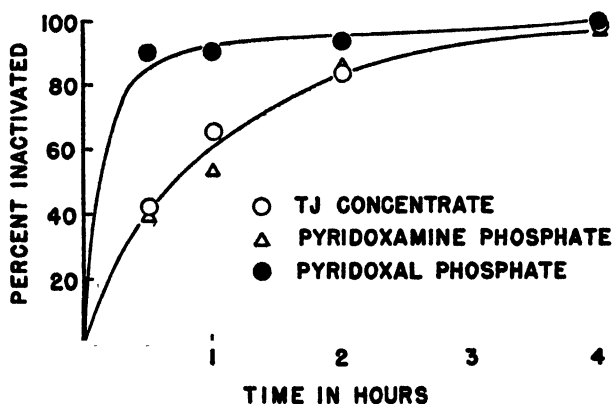


FIG. 1. Assay by Procedure B of aliquots of pyridoxamine phosphate, pyridoxal phosphate, and TJ concentrate.

These microbiological and chemical data demonstrate that the TJ activity of crude materials is due to their content of pyridoxamine phosphate and pyridoxal phosphate. D-Alanine may be presumed to be an essential metabolite for *L. lactis*, as has been demonstrated for *L. casei*, *Streptococcus faecalis*, and *Lactobacillus delbrueckii* (7, 9). *L. lactis*, like *L. acidophilus* and *L. helveticus*, utilizes the phosphorylated forms of vitamin B₆ more effectively.

SUMMARY

1. Microbiological evaluation of crude materials under several different experimental conditions and with several lactic acid bacteria indicates that the TJ activity of these materials is due to pyridoxamine phosphate and pyridoxal phosphate.

2. Pyridoxamine phosphate is the most active of the B₆ vitamins in

supporting the growth of *Lactobacillus lactis*, *Lactobacillus helveticus*, and *Lactobacillus acidophilus*. Pyridoxal phosphate, which may be converted to pyridoxamine phosphate under certain conditions, is approximately one-third to one-twentieth as active, depending upon the test organism. Much higher concentrations of pyridoxal, pyridoxamine, pyridoxine, and D-alanine are required for growth.

3. Microbiological and chemical data indicate that the activity of a TJ concentrate obtained from *Streptomyces griseus* fermentations is due to its pyridoxamine phosphate content.

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THE EFFECT OF ANIONS ON THE ACTIVITY OF CARBOXYPEPTIDASE*

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It was recently reported by Smith and Hanson (2, 3) that the enzymatic activity of carboxypeptidase is inhibited by certain anions, such as orthophosphate, pyrophosphate, citrate, and oxalate. From these findings and from the observation of spectroscopically detectable quantities of magnesium in several times recrystallized preparations of the enzyme (2), it was concluded that carboxypeptidase is a metal protein (4). It was further postulated that the metal, magnesium in this case, is capable of combining through two coordinate bonds with structural elements on either side of the susceptible bond of the substrate in the formation of a seven-membered chelate ring which contributes to the total catalytic effect of the enzyme by its electronic pull on the elements of the hydrolyzable bond (4-6).

In the absence of quantitative analytical data for the magnesium content of carboxypeptidase, the experimental evidence for the metal activation of this enzyme rests almost exclusively on the inhibitory effect of certain anions. It was deemed of importance, therefore, to repeat these experiments of Smith and Hanson (3) in greater detail. Our inquisitiveness was elicited by considerations of both experimental and theoretical nature, the more important of which may be stated as follows: (1) The published experimental data were obtained with the use of carbobenzoxyglycyl-L-leucine (CGL) as substrate rather than with the more susceptible substrate (7) carbobenzoxyglycyl-L-phenylalanine (CGP). Moreover, on the basis of the rate value of Stahmann, Fruton, and Bergmann (8) for the hydrolysis of CGL, the preparation of carboxypeptidase used in the work of Smith and Hanson contained only about 40 per cent of active enzyme. (2) Unlike crystalline enolase (9), which is a typical magnesium-activated enzyme, carboxypeptidase is not inactivated by fluoride, alone or in combination with 0.01 M orthophosphate (3). (3) Relatively high concentrations and prolonged incubation of orthophosphate, citrate,

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and oxalate were required to produce a significant degree of inhibition. (4) The stoichiometry of inhibition was evaluated from interpretations of hydrolysis rates, measured at a single initial substrate concentration, by first order reaction kinetics to which these systems allegedly adhered rigidly. The inherent ambiguity of such interpretations has been adequately considered elsewhere (10).

In the work presented herein, particular emphasis was laid on the determination of *initial* reaction velocities (10). Rate measurements were conducted at several initial substrate concentrations and were interpreted by the kinetic constants, K_m and k_3 , the Michaelis constant and the specific rate of activation, respectively (10). The test substrate was initially carbobenzoxyglycyl-L-phenylalanine (CGP), but for purposes of strict comparison with Smith and Hanson's experiments, measurements with carbobenzoxyglycyl-L-leucine were also included and the kinetic constants for the hydrolysis of this substrate were determined.

The data presented herein fail to provide evidence for the metal activation of carboxypeptidase.

EXPERIMENTAL

Materials and Methods

Six times recrystallized preparations of carboxypeptidase, prepared by methods described elsewhere (10, 11), were employed.¹

Carbobenzoxyglycyl-L-phenylalanine (CGP) was prepared as previously described (11).

Carbobenzoxyglycyl-L-leucine (CGL) was prepared by Mr. Jules A. Gladner of this Laboratory by the method given by Stahmann, Fruton, and Bergmann (8). After repeated recrystallizations, the melting point remained constant at 102–103°, in contradistinction² to the reported value (8) of 141–142°.

$C_{16}H_{25}O_4N_2$. Calculated. C 59.6, H 6.9, N 8.69

322.4 Found.³ " 59.55, " 6.9, " 8.66

$[\alpha]_D^{25} = -10.1$ (5% in ethyl alcohol); reported (8) -10.3 .

On hydrolysis with carboxypeptidase, the preparation yielded the theoretical amount of leucine.

Rates of hydrolysis, at 25°, were determined with the colorimetric ninhydrin method of Moore and Stein (12), as recently described (13), after

¹ We are indebted to Mr. J. H. Weare and Dr. J. B. Lesh of the Armour Laboratories for the supply of freshly frozen exudate of beef pancreas glands.

² This discrepancy of melting points cannot be explained on the basis of the published data.

³ Chemical analyses by the Elek Micro Analytical Laboratories, Los Angeles, California.

calibration for the color yield of phenylalanine and leucine, respectively. Initial reaction velocities were determined from the *initial* slopes when the data were plotted according to the first order reaction equation.

Results

Carbobenzoxyglycyl-L-phenylalanine (CGP)

It has been demonstrated in recent work that, within the range of an initial substrate concentration of 0.02 to 0.07 M, the hydrolysis of CGP by carboxypeptidase follows the integrated Michaelis-Menten equation (7, 10). Within this concentration range, a plot of hydrolysis rates according to first order reaction equation yields curves which are either apparently linear or convex toward the axis of the abscissas. Since the experiments from which these conclusions were derived have been questioned (3) on the grounds that orthophosphate was employed as buffer (0.04 M), the kinetics of the hydrolysis of CGP in the absence of orthophosphate were investigated, with a 0.04 M veronal buffer, pH 7.5 (0.0114 M NaV, 0.0286 HV), containing also 0.1 M LiCl. The shapes of the hydrolysis curves were the same as those previously given for the hydrolysis in the presence of 0.04 M phosphate buffer, pH 7.5, containing 0.1 M LiCl. A plot of the data according to one of the equations of Lineweaver and Burk (14) is shown in Fig. 1. The kinetic constants, K_m and k_2 , listed in the legend to Fig. 1 are practically the same as those previously reported (7).

Orthophosphate—In the presence of 0.2 M orthophosphate buffer, pH 7.5, a plot of hydrolysis rates according to the first order reaction equation showed a negative deviation from linearity at an initial substrate concentration (0.04 M) in which otherwise positive deviations have been observed. This result has been observed regardless of whether orthophosphate was added immediately preceding the start of hydrolysis or whether, previous to the kinetic determination, enzyme and 0.2 M orthophosphate were incubated for 15 hours in the cold. This apparently rate-retarding effect of orthophosphate is thus an immediate one and may be immediately reversed by dilution. Thus, if prior to the addition of the substrate the phosphate concentration is reduced to 0.04 M, and 0.1 M LiCl is added, the shape and initial slope of the rate curve revert to that normally obtained.

A typical kinetic experiment in the presence of 0.2 M orthophosphate is shown in Fig. 2. The initial velocity, calculated from experiments in veronal buffer, is given by the straight thin line and coincides reasonably well with the tangent to the curve at zero time. When the initial velocity was similarly determined for measurements performed over a range of initial substrate concentrations, the results given in Fig. 1 were obtained. The data given in the legend to Fig. 1 indicate that the kinetic

constants, K_m and k_2 , are practically independent of the presence of orthophosphate and of its concentration, provided that the *initial* reaction velocity is used as a measure of hydrolysis rates (10).

The shape of the hydrolysis curves obtained in the presence of 0.2 M orthophosphate suggested that in the presence of this ion the reaction is inhibited by one of the reaction products (10). In order to test this hypothesis, experiments were performed in which carbobenzoxyglycine and L-phenylalanine, respectively, were added to the reaction mixture. While carbobenzoxyglycine was without demonstrable effect, L-phenylalanine decreased appreciably even the *initial* reaction velocity, as shown

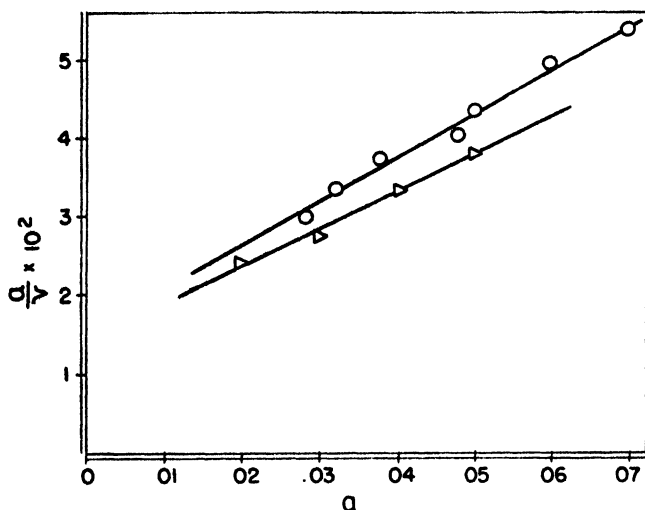


FIG. 1. A plot of the dependence of the initial reaction velocity, v , on initial substrate concentration, α , for the hydrolysis of CGP by carboxypeptidase. O in veronal buffer, pH 7.5; Δ in 0.2 M orthophosphate buffer, pH 7.5. The calculated kinetic constants were as follows: veronal, $K_m = 0.027$ M, $k_2 = 1.8$; 0.04 M orthophosphate, $K_m = 0.028$ M, $k_2 = 2.1$; 0.2 M orthophosphate, $K_m = 0.030$ M, $k_2 = 2.1$.

in Fig. 2. A similar decrease in the initial reaction velocity by L-phenylalanine was observed when the initial substrate concentration was varied. The inhibitory effect of L-phenylalanine could account for the shape of the hydrolysis curve observed in the presence of 0.2 M orthophosphate. In fact, the solid curve connecting the experimental points in Fig. 2 was calculated on that assumption (equation (37) of Neurath and Schwert (10)) by means of the K_i value for L-phenylalanine determined as described later in this paper.

In the presence of 0.2 M orthophosphate, as under conditions previously described (0.04 M orthophosphate (15)), the inhibition by L-phenylalanine

is negligible compared to that exerted by the *D* isomer. Thus, quantitative measurements in which 0.0215 *M* *D*-phenylalanine was added yielded $K_i = 1.6 \times 10^{-3}$ *M*, as compared to $K_i = 2.0 \times 10^{-3}$ *M* previously reported (15). Similar measurements in the presence of 0.2 *M* orthophosphate in which 0.0406 *M* *DL*-phenylalanine was added yielded $K_i = 4.0 \times$

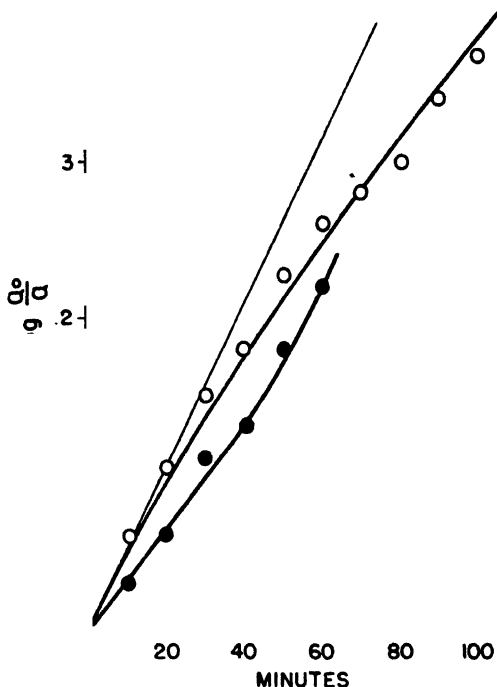


FIG. 2. A plot of the hydrolysis of CGP by carboxypeptidase according to first order reaction kinetics in the presence of 0.2 *M* orthophosphate buffer, pH 7.5. Initial substrate concentration, 0.04 *M*. O without added *L*-phenylalanine; ● in the presence of added *L*-phenylalanine (0.022 *M*). The continuous curved line has been calculated on the basis of competitive inhibition (equation (37) of Neurath and Schwert (10)), with $K_m = 0.03$ *M*, and $K_i = 0.001$ *M*. The tangential straight line represents the initial velocity calculated from rate measurements in the absence of orthophosphate (veronal buffer).

10^{-3} *M* on the basis of the concentration of the racemate or $K_i = 2.0 \times 10^{-3}$ *M* when referred to the concentration of the added *D*-amino acid.

Pyrophosphate—In the presence of 0.01 *M* pyrophosphate which, according to Smith and Hanson (3), causes 100 per cent inhibition of the hydrolysis of CGL, the inhibition by liberated *L*-phenylalanine is so pronounced that the initial velocity of the hydrolysis of CGP cannot be determined with the required precision. In these experiments, 0.01 *M* sodium

pyrophosphate was added to the 0.04 M orthophosphate buffer, pH 7.5. Representative results are given in Fig. 3. However, the inhibitory effect can be fully and instantaneously reversed by 100-fold dilution of the enzyme-pyrophosphate mixture. This is shown by the straight line of Fig. 3 which agrees reasonably well with the tangent to the concave curve at zero time, indicating that pyrophosphate is also without effect on the initial hydrolysis rate.

Other Anions—Sodium sulfate (0.1 M), sodium citrate (0.1 M), and sodium oxalate (0.1 M) were without effect on the initial hydrolysis or on

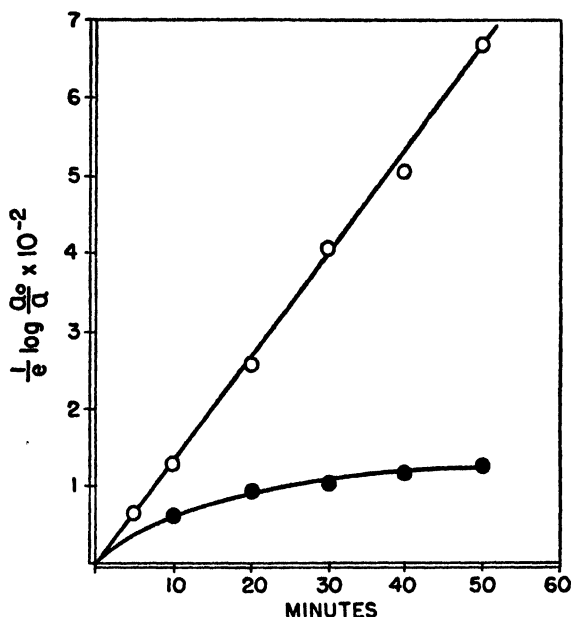


FIG. 3. A plot of the hydrolysis of CGP by carboxypeptidase, according to first order reaction kinetics (corrected to unit enzyme N concentration), in the presence of 0.01 M pyrophosphate (O) and after reversal of pyrophosphate inhibition by 100-fold dilution (●). All measurements in 0.04 M orthophosphate buffer, pH 7.5.

the shape of the hydrolysis curves, even after 15 hours of incubation with the enzyme in the cold. In all these measurements, controls for possible partial inactivation of the enzyme alone were made, and where necessary, the proper corrections were applied. Representative data are given in Fig. 4 from which the positive deviations from the semilogarithmic plots, and hence the lack of inhibition by the reaction products, are evident. When corrected for differences in the initial substrate concentration and enzyme concentration, the initial hydrolysis rates represented by the curves in Fig. 4 are identical.

In the presence of 0.002 M sodium cyanide which was reported to cause more than 50 per cent inhibition of the hydrolysis of CGL, the initial hydrolysis rate of CGP was practically unaffected, k_2 being 2.0 as compared to 2.1 in 0.04 M orthophosphate alone. The shapes of the hydrolysis curves failed to reveal evidence of inhibition by reaction products.

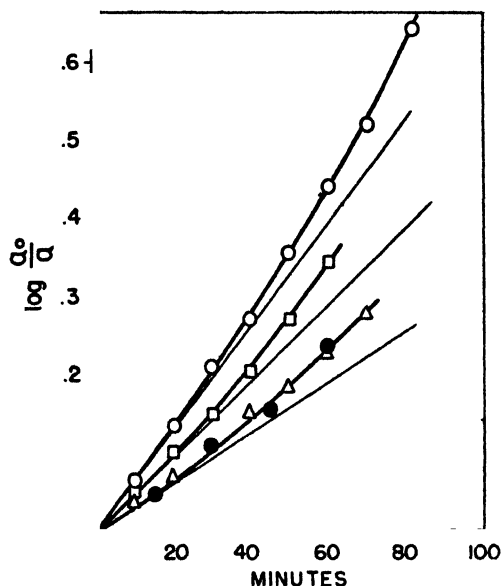


FIG. 4. Effect of various anions on the shapes of the hydrolysis curves of CGP, plotted according to first order reaction kinetics. The initial reaction velocity is indicated by the initial tangents. Enzyme concentration, 4×10^{-4} mg. of enzyme N per cc. in all cases except for measurements with oxalate (3.7×10^{-4} mg. per cc.). \circ 0.1 M sulfate, 0.04 M orthophosphate buffer, 0.04 M CGP; \triangle 0.1 M citrate, 0.04 M orthophosphate buffer, 0.059 M CGP; \square 0.1 M oxalate, 0.04 M orthophosphate buffer, 0.039 M CGP; \bullet 0.04 M veronal buffer, 0.1 M LiCl, 0.07 M CGP.

Carbobenzoxylglycyl-L-leucine (CGL)

The kinetics of the hydrolysis of this substrate in veronal buffer, pH 7.5, containing 0.1 M LiCl, was determined in the usual manner and the kinetic constants K_m and k_2 were calculated from the plot shown in Fig. 5. Similar measurements, carried out in the 0.04 M orthophosphate buffer, pH 7.5, containing 0.1 M LiCl, also shown in Fig. 5, yielded constants insignificantly different from those obtained in the absence of orthophosphate. No significant differences in the shapes of the hydrolysis curves were observed as compared to the measurements in veronal buffer.

Comparison of these kinetic constants with those for the hydrolysis of

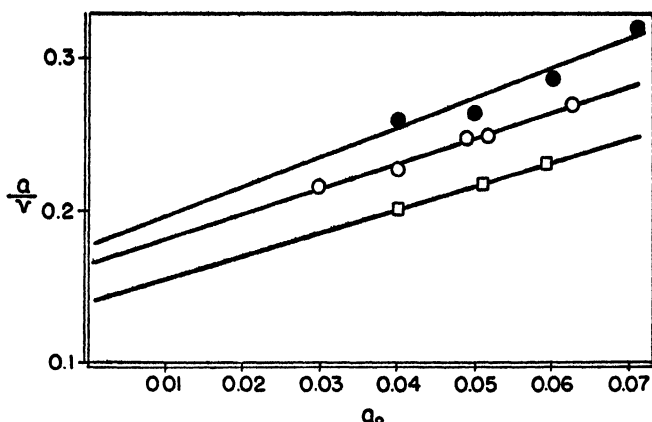


FIG. 5. A plot of the dependence of the initial reaction velocity, v , on initial substrate concentration, a_0 , for the hydrolysis of CGL by carboxypeptidase. \square in veronal buffer; \circ in 0.04 M orthophosphate buffer; \bullet in 0.2 M orthophosphate buffer. All measurements at pH 7.5. The calculated kinetic constants were as follows: veronal, $K_m = 0.093$ M, $k_2 = 0.67$, $C_{max.} = 3.1$; 0.04 M orthophosphate, $K_m = 0.097$ M, $k_2 = 0.60$; 0.2 M orthophosphate, $K_m = 0.090$ M, $k_2 = 0.51$.

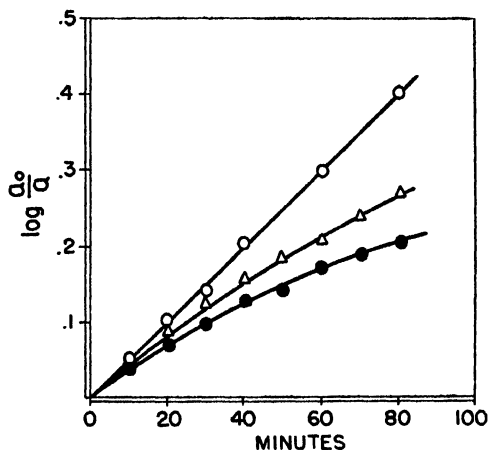


FIG. 6. Effect of various anions on the shapes of the hydrolysis curves of CGL, plotted according to first order reaction kinetics. Initial substrate concentration approximately 0.05 M, enzyme concentration approximately 2.5×10^{-3} mg. of enzyme N per cc. \circ veronal buffer; \triangle 0.04 M orthophosphate buffer containing 0.1 M citrate; \bullet 0.2 M orthophosphate buffer. Since the hydrolysis curve obtained in 0.04 M orthophosphate buffer containing 0.1 M oxalate was directly superimposable on the 0.2 M orthophosphate curve, the experimental points have been omitted for purposes of clarity.

CGP (7, 10) indicates that both the enzyme-substrate affinity and the specific rate of activation for the leucyl peptide are lower than for the

analogous phenylalanyl peptide. The maximum proteolytic coefficients (10) have a ratio of about 1:9. The apparent proteolytic coefficient in 0.05 M initial substrate concentration is 2.0, as compared to the value of 2.6 reported by Stahmann *et al.* (8), whereas the corresponding value given by Smith and Hanson (3) is 0.9. It appears, therefore, that the preparation used by the latter authors was only 35 to 45 per cent active.

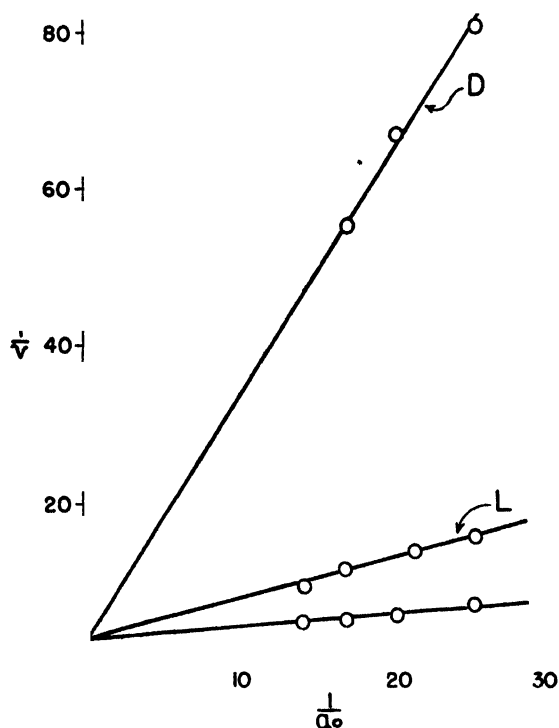


FIG. 7. A plot to demonstrate competitive inhibition of the hydrolysis of CGL by 0.02 M D- and L-phenylalanine, respectively. Measurements in the presence of 0.2 M orthophosphate buffer, pH 7.5.

Orthophosphate—In the presence of 0.2 M orthophosphate, pH 7.5, the shapes of the hydrolysis curves are indicative of inhibition by reaction products, as shown in Fig. 6. As in analogous measurements involving CGP as substrate, the tangent to the initial slope coincides closely with the straight line obtained in the absence of orthophosphate (veronal buffer). A plot of the data from which K_m and k_s were calculated is included in Fig. 5. A small variation of k_s and K_m with orthophosphate concentration (see the legend to Fig. 5) though not self-consistent, is evident.

Since the measured degree of inhibition by a competitive inhibitor is

increased as K_m increases,⁴ the inhibitory activity of L-phenylalanine and the effect of orthophosphate thereon were determined by quantitative measurements with use of CGL rather than CGP as substrate. The results of measurements performed in 0.2 M orthophosphate, pH 7.5, are shown in Fig. 7 in which the reciprocal of the initial velocity is plotted against the reciprocal of the initial substrate concentration. The straight line, obtained in the presence of 0.02 M L-phenylalanine, extrapolates to the same ordinate intercept as that obtained in the absence of added amino acid, indicative of competitive inhibition. Analogous measurements for the inhibition by 0.02 M D-phenylalanine are likewise plotted in Fig. 7 and demonstrate the considerably higher inhibitory activity of the unnatural isomer. The calculated K_i values are given in Table I and indicate an approximate ratio of K_i (L form) to K_i (D form) of 9. The re-

TABLE I
*Inhibition by Phenylalanine**

Isomeric form	Orthophosphate	Substrate	K_i
	M		10^{-3} M
D	0.04	CGP	2.0 (cf. (15))
L	0.04	CGL	17.5
D	0.2	CGP	1.6
DL	0.2	"	1.8†
D	0.2	CGL	1.2
L	0.2	"	9.8

* CGP denotes carbobenzoxyglycyl-L-phenylalanine, CGL carbobenzoxyglycyl-L-leucine. All measurements at pH 7.5, 25°.

† This value was calculated from the concentration of the D isomer, assuming the inhibition by the L form to be insignificant in comparison (see the text).

sults of measurements of the dependence of K_i of L-phenylalanine on orthophosphate concentration are plotted in Fig. 8. The K_i values of D- and L-phenylalanine, calculated from inhibition measurements performed under various conditions, with CGP and CGL as substrate, respectively, are summarized in Table I.

While the absolute K_i values are dependent on orthophosphate concentration, particularly when CGL is used as substrate, the ratio of K_i (L form) to K_i (D form) is nearly independent of the concentration of this anion.

Other Anions—The effects of 0.1 M oxalate and 0.1 M citrate, respectively, on the shapes of the hydrolysis curves, measured in the presence

⁴ It was erroneously stated in a recent discussion of this problem that inhibition decreases as K_m : K_i increases ((10) p. 92).

of 0.04 M orthophosphate, are indicated by Fig. 6 in which, for comparison, the results of measurements in the presence of veronal buffer alone are also plotted. The initial slopes of the rate curves are unaffected by oxalate and citrate but, in contrast to analogous measurements of the hydrolysis of CGP, a gradual retardation of hydrolysis rates, presumably by inhibition by L-leucine, is evident.

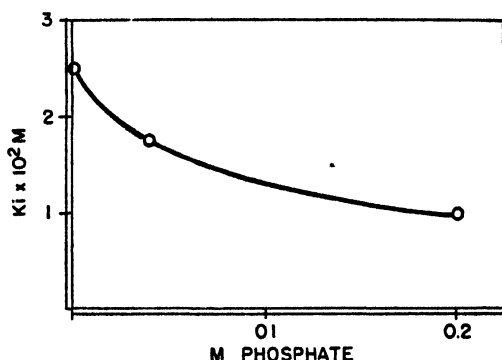


FIG. 8. A plot of K_i of L-phenylalanine, determined from measurements of the hydrolysis of CGL, against orthophosphate concentration.

DISCUSSION

The results of the present investigation demonstrate clearly that the *initial* velocity of the hydrolysis of the substrates CGP and CGL by carboxypeptidase is unaffected by orthophosphate, citrate, oxalate, and cyanide, and apparently also unaffected by pyrophosphate. It may be concluded, therefore, that these anions have no effect, directly or indirectly, on the enzymatic activation process. In the presence of these anions, however, competitive inhibition by the liberated amino acid may ensue. The competitive inhibition by L-phenylalanine in the presence of 0.2 M orthophosphate has been established by three observations: (1) the adherence to the differential velocity equation for competitive inhibition (Fig. 7), (2) the close agreement between the experimental points for the hydrolysis of CGP and the rate curve calculated from the integrated velocity equation (Fig. 2), and (3) the approximate independence of the calculated K_i value for L-phenylalanine of the nature of the substrate (CGP and CGL, respectively). The effect of pyrophosphate on the shape of the rate curves may be analogously attributed to inhibition by the free amino acid.

The present findings fail to provide evidence for a binding of these anions by metal coordination. Their effects on inhibition by the reaction product are experimentally instantaneous, fully reversible, and moreover,

require relatively high concentrations of these salts. These findings are interpretable, however, by the general phenomenon of anion binding by proteins (16), and, in this specific case, may be ascribed to the electrostatic binding by a positively charged group of the active center of carboxypeptidase.

It has been shown by Elkins-Kaufman and Neurath (7, 15) that in the presence of 0.04 M orthophosphate buffer, pH 7.5, the rate of desorption of L-phenylalanine is not a rate-determining step in the hydrolysis of CGP by carboxypeptidase. However, when the cationic charge of the enzyme is decreased by a shift to pH 9.0, marked inhibition by L-phenylalanine ensues. A masking of the postulated positive charge of the active center (15) by electrostatic anion binding rather than by proton donation would obviously produce the same result. The findings that the ratio of the K_i values for the optical isomers of phenylalanine is approximately independent of the concentration of orthophosphate, although the absolute values are concentration-dependent, are in full accord with previous views on the relative contributions of electrostatic repulsive forces to the interaction of carboxypeptidase with D- and L-phenylalanine, respectively (15). While in the absence of such repulsive forces the L isomer should be the better inhibitor, it must be remembered that the shielding of the positive charges by the interposition of an anion will increase by several angstrom units the distance of closest approach of the amino acid to the enzyme and thus decrease the total binding.

In the light of the present experiments, the hypothesis of the metal activation of carboxypeptidase appears entirely devoid of experimental evidence.

This work has been supported in part by the United States Public Health Service, National Institutes of Health.

SUMMARY

In view of recent reports of Smith and Hanson (2, 3) on the metal activation of carboxypeptidase, the effects of certain anions on the enzymatic hydrolysis of carbobenzoxyglycyl-L-phenylalanine and of carbobenzoxyglycyl-L-leucine were reinvestigated. None of the following anions had a significant effect on the initial hydrolysis rate of these substrates: orthophosphate, pyrophosphate, oxalate, citrate, and cyanide. It has been concluded, therefore, that these anions have no effect, directly or indirectly, on the enzymatic activation process, and that the hypothesis of the metal activation of carboxypeptidase is devoid of experimental evidence.

In the presence of some of these anions, *e.g.* orthophosphate and pyrophosphate, the shapes of the hydrolysis curves are altered as a result of competitive inhibition by the liberated amino acid. The enzyme-inhibitor dissociation constant, K_i , for L-phenylalanine has been determined from measurements in the presence of varying concentrations of orthophosphate and found to be about 9 times greater than that of the D isomer.

The present findings are in full accord with previous views (7, 10, 15) on the specificity and mode of action of carboxypeptidase.

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ENZYMIC OXIDATION OF FORMIC ACID*

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Interest in the metabolic fate of formic acid has been renewed as the result of recent experiments showing active incorporation of carbon-labeled formate into normal body constituents of the pigeon (1-3) and the rat (4, 5). Prior to these findings, enzymes capable of catalyzing the oxidation of formic acid had been reported, widely distributed in plant and animal tissues (6).

Only in the case of a preparation from green peas was the reaction well defined, however. Adler and Sreenivasaya (7) demonstrated that direct reduction of diphosphopyridine nucleotide (DPN) by formate was catalyzed by dialyzed pea extracts. This finding extended the work of Andersson (8) who had shown that DPN was necessary for the reduction of methylene blue by formate in such preparations.

The object of the present study was to obtain additional information about enzyme systems capable of oxidizing formic acid. Studies on the enzyme from green peas were therefore extended. Attempts to demonstrate the presence in animal tissues of soluble enzymes capable of oxidizing formate were also made and were successful in the case of rat liver and kidney.

EXPERIMENTAL

Materials and Methods—Green peas of the previous year's harvest (known as "Laxton's progress peas") were purchased from Vaughan's Seed Store, Chicago. Cozymase was obtained in several lots from the Schwarz Laboratories. As determined by the sodium borohydride procedure (9), the range of purity was 39 to 44 per cent. The sodium salts of adenosine-triphosphate (ATP) and adenosinemonophosphate (muscle adenylic acid, AMP) were prepared, respectively, from the barium salt of ATP and from the free acid AMP purchased from the Sigma Chemical Company.

Rates of oxygen uptake were measured in the Warburg apparatus at 30°. Spectrophotometric experiments were carried out with the Beck-

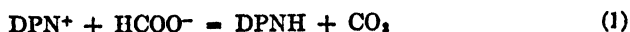
* This work was supported by a grant to Dr. T. R. Hogness from the Rockefeller Foundation.

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man quartz spectrophotometer at 30°, with a maximum variation of 1.0° in the temperature of the cell contents during the course of any single test.

Protein dry weights were determined by evaporation of 0.5 cc. of dialyzed solution for 1 hour at 110°, followed by 3 hours further heating at the same temperature.

Measurement of Enzyme Activity—As will be shown later, it was possible to fractionate the preparations employed by previous investigators and show that two enzymes were responsible for the reduction of methylene blue by formate in the presence of pea extracts. One of these, formic dehydrogenase,¹ catalyzes the reaction



The other catalyzes the oxidation of reduced DPN by methylene blue and is referred to as the DPNH-oxidizing activity.

Formic dehydrogenase was assayed by determination of the rate of reduction of DPN by added formate. The formation of reduced DPN (DPNH) was measured by observing the increase in light absorption at 340 mμ. In the presence of an excess of formate the reaction followed first order kinetics with respect to DPN.

Since the optical density ($\log I_0/I$) at 340 mμ is proportional to the concentration of DPNH, we may write

$$\log (D_0/D_0 - D) = K't \quad (2)$$

where D_0 is the calculated optical density when all the DPN is reduced, and D is the measured optical density at time t (in minutes) from the beginning of the reaction. A plot of the left-hand side of Equation 2 against t (in minutes) yields a straight line with a slope equal to K' (Fig. 1). Since under the experimental conditions chosen the value of K' is proportional to the weight of protein taken for the test, the activity (W) of a preparation is defined as $W = K'$ per gm. of protein under the conditions of the test, as given in Fig. 1.

The assay of DPNH-oxidizing activity was based upon the observation of the rate of decrease of optical density at 340 mμ when methylene blue (MB) was added to DPNH in the presence of the enzyme. This reaction also showed apparent first order kinetics with respect to DPN.

In this case the DPN is present as DPNH at the beginning of the test, and

$$\log D_0/D = k't \quad (3)$$

¹ The term formic dehydrogenase has been heretofore loosely applied in connection with a variety of biological reactions involving formic acid. Use of this term will be restricted subsequently to the specific enzyme-catalyzed reaction between formate and DPN according to the equation $\text{DPN}^+ + \text{HCOO}^- = \text{DPNH} + \text{CO}_2$.

where D_0 represents the optical density at the start of the measurement and D is the optical density at time t (in minutes). A plot of $\log D$ against t (in minutes) yields a straight line with a slope equal to $-k'$ (Fig. 2). Since under the experimental conditions chosen the value of k' is found to

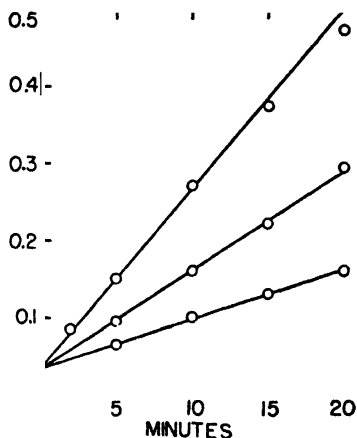


FIG. 1

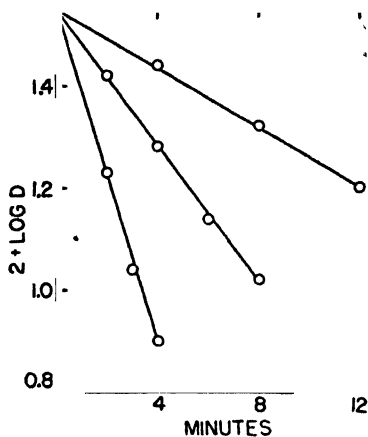


FIG. 2

FIG. 1. Reduction of DPN by formic dehydrogenase. Top curve, 0.1 cc. of enzyme ($K' = 0.0245$); middle curve 0.05 cc. of enzyme ($K' = 0.0132$); bottom curve 0.025 cc. of enzyme ($K' = 0.0061$). The last two points were not utilized in drawing the top curve. The departure from linearity was due to the enzymic destruction of DPN, which will be discussed later. The test was carried out in two cuvettes 1 cm. in length, the reference cell containing all additions except that of DPN. The contents of the reaction cell, which were equilibrated to 30° before the final addition of enzyme, were as follows: 1.00 cc. of 0.5 M phosphate buffer, pH 8.0, 0.25 cc. of 0.5 M sodium formate, 0.10 cc. of 0.0024 M DPN, 1.60 cc. of distilled water.

FIG. 2. Oxidation of DPNH. Top curve, 0.05 cc. of enzyme ($k' = 0.032$); middle curve, 0.1 cc. of enzyme ($k' = 0.071$); bottom curve, 0.20 cc. of enzyme ($k' = 0.148$). When the reoxidation of DPNH is 50 per cent complete within 2 minutes, as shown in the bottom curve, the assay for enzyme activity has only limited accuracy. For practical measurements, the time required for 50 per cent reoxidation should not be less than 3 minutes or more than 12 minutes. The test was carried out in two cuvettes 1 cm. in length, the reference cell containing all additions except that of DPNH. The contents of the reaction cell, which were equilibrated to 30° before final addition of enzyme, were as follows: 0.05 cc. of DPNH solution (described below), 1.00 cc. of 0.5 M sodium phosphate buffer, pH 8.0, 1.75 cc. of distilled water, 0.1 cc. of 0.0054 M MB.

be proportional to the weight of protein taken in the test, the activity (U) of a preparation is defined as $U = k'$ per gm. of protein under the conditions of the test, as given in Fig. 2.

DPNH prepared by reduction with sodium borohydride (9) or with sodium hydrosulfite (10) proved unsatisfactory for use in the above test be-

cause of instability on storage and reoxidation in the absence of enzyme. When DPNH was prepared by enzymic reduction, it gave a negligible oxidation blank and could be stored satisfactorily in the frozen state for at least 1 month. 25 mg. of DPN, 1.50 cc. of 0.5 M Na_2HPO_4 , 0.40 cc. of enzyme, and 0.60 cc. of 0.5 M sodium formate were incubated at 30°. After 5 hours, 0.50 cc. of 0.50 M NaOH were added to give a pH, when diluted, of 10.0 to 10.5. The mixture was centrifuged and the supernatant stored at -15°. Reduction of DPN was about 80 per cent complete.

In the above test it was necessary to correct observed values of optical density by a residual optical density obtained after complete oxidation of DPNH. The residual optical density varied from an initial value of 0.05 for a freshly prepared DPNH solution to as high as 0.15 for the same solution after storage for 1 month.

Three procedures were used to obtain the residual optical density, and all gave values in close agreement with each other. When the oxidation was rapid and there was only inappreciable destruction of DPN, the residual optical density was directly observed. In other cases, additional enzyme was added to accelerate the rate of oxidation. Finally, the addition of acetaldehyde in conjunction with the alcohol dehydrogenase, present in many of the enzyme fractions, brought about very rapid oxidation of DPNH.

Both assays have the disadvantage that they are inaccurate in the presence of enzymes which destroy DPN. This effect will be discussed in detail below.

Results

Fractionation of Enzyme Components from Peas—In their previous studies, Adler and Sreenivasaya (7) extracted formic dehydrogenase from peas with 0.1 M Na_2HPO_4 . They concentrated and purified the preparation by one precipitation with ammonium sulfate, evaporation *in vacuo*, and dialysis. The activity was measured by the determination of reduction time of methylene blue in the presence of added formate.

In the present studies, the purification of the formic dehydrogenase was carried further by the following procedure. 5 kilos of peas were soaked in tap water at room temperature for 15 hours. The swelled peas were blended with an equal weight of 0.1 M Na_2HPO_4 in a Waring blender and kept at room temperature, with occasional stirring, for 2 to 3 hours. The blend was pressed through heavy toweling by means of a hand press, and the fluid centrifuged for 1.5 hours at $2500 \times g$ in a 13 liter International serum centrifuge. The yield was 12 liters of clear yellow supernatant liquid, with a dry weight of 1 kilo, $W = 0.3$, $U = 1.8$.

To each liter of crude extract 220 gm. of solid ammonium sulfate were

added and brought into solution. The mixture was kept at room temperature for 1 hour before being centrifuged at $2500 \times g$ for 1.5 hours. The supernatant was treated with an additional 120 gm. of ammonium sulfate per liter of original crude extract, kept at room temperature for 2 hours, and centrifuged for 1 hour. The residue was dissolved in about 600 cc. of distilled water, placed in cellophane sacs, and dialyzed with constant agitation against running distilled water at 4° for 24 hours. A considerable precipitate of inert protein was removed by centrifugation to yield 800 cc. of clear yellow solution, Fraction P8, containing 45 gm. of protein, $W = 4.0$, $U = 10$.

Further fractionation was carried out with additions of small increments of ammonium sulfate. After each addition the mixtures were allowed to stand 12 hours before centrifugation. The results of this second fractionation appear in Table I.

The maximum purification achieved over the activity of the initial extract was 27-fold for formic dehydrogenase and 69-fold for the DPNH-

TABLE I
Fractionation of Extract of Peas, Fraction P8

Fraction No. (NH_4) $_2$ SO $_4$ added, gm. per 100 cc. Dry weight, gm.	1	2	3	4	5	6	7	8	9
	20	3	3	3	3	3	3	5	10
	6.4	2.6	4.6	2.8	3.6	1.1	1.0	1.4	0.8
W	2.4	5.0	5.8	8.0	6.0	0	0	0	0
U	6	4	5	17	32	124	78	35	18

oxidizing activity. The conclusion that formic dehydrogenase activity and the DPNH-oxidizing activity are not associated in one protein molecule is supported by the separations obtained.

Some Properties of Formic Dehydrogenase System—The formic dehydrogenase activity was precipitated mainly in the range of 23 to 32 gm. of ammonium sulfate per 100 cc. Continued fractionation was accompanied by relatively large losses of activity, with no compensating increase in purity of the enzyme.²

² Fractional precipitations at 0° and at pH 6.0 and 7.0 were carried out by carefully controlled additions in separate experiments of solid ammonium sulfate, neutral saturated ammonium sulfate solution, and ethyl alcohol. It was observed in every case that clear supernatants could not be obtained by centrifuging and that all of the enzyme activity initially present was not recovered. The formation of colloidal solutions was particularly marked at pH 7.0, when little protein could be recovered below 70 per cent saturation with ammonium sulfate or below an alcohol concentration of 65 per cent by weight. Recovery was much improved at pH 6.0, but instability of the enzyme precluded extension of the investigation to lower pH values. Similar diffi-

The initial extract (Fraction P8) contained both formic dehydrogenase activity and DPNH-oxidizing activity. The addition of DPN, sodium formate, and MB to this preparation led to an oxygen consumption (measured manometrically) which continued at a constant rate for several hours. The composition of the complete system and the effect of varying the concentrations of the components of the system are presented in Table II. In similar experiments it was possible to cause a 50 per cent increase in the rate of oxygen uptake of Fraction P8-3 by the addition of Fraction P8-7,

TABLE II
Dependence of Rate of Oxygen Uptake upon Concentration of Each Component of Formic Acid-Oxidizing System

Amount of component added (1)	0.5 M formate (2)	0.0024 M DPN (3)	0.50 per cent MB (4)	Enzyme (5)	Optimum rate per cc. enzyme (6)
cc.					
0	8	8	4	0	
0.02			51		
0.03	36				
0.05	47	39	50		
0.10			65		
0.15	70	65	73		
0.25	73	70	73	40	160
0.30	74	73			
0.50		76		70	140
0.75				98	130
1.00				130	130

Except for the variations indicated in the table the reacting system consisted of 0.25 cc. of 0.0024 M DPN, 1.00 cc. of 0.5 M phosphate buffer, pH 8.0, 0.2 cc. of 0.5 per cent MB, 0.25 cc. of 0.5 M sodium formate, 0.5 cc. of enzyme, distilled water to make 3.00 cc., and 0.1 cc. of 20 per cent KOH in the center well of the Warburg vessel. All results are expressed as microliters of O_2 per hour. Columns 2 to 5 each show a series of experiments in which the component listed at the head of the column was added in the different amounts indicated in Column 1, while all other components were kept constant.

a preparation containing the DPNH-oxidizing activity but no formic dehydrogenase. The stimulating effect was even more marked when Fraction P8-3A was combined with Fraction P8-7 (Table III). Frac-

culties were encountered by Stumpf (11) in the purification of aldolase from green peas. He reported that a maximum purification limit (about 92-fold over the initial extract) was quickly reached and that all further attempts at fractionation by the addition of either ammonium sulfate or of acetone led to colloidal solutions which did not give clear out fractions.

tion P8-3A was obtained by two successive refractionations of Fraction P8-3, with selection each time of the precipitate obtained in the range of 20 to 26 gm. of ammonium sulfate per 100 cc. of original solution.

By means of the spectrophotometric test the reduction of DPN was found to be more than 95 per cent inhibited by 0.001 M HCN, whereas this concentration of HCN had no measurable effect on the rate of oxidation of DPNH by the same enzyme preparation. The activity of the enzyme was unaltered in the presence of 0.001 M ATP.

The formic dehydrogenase activity was preserved with little loss for 3 months as a lyophilized powder or in the frozen state at -15° . At 4° most of the activity disappeared within 1 week. The enzyme lost 50 per cent of its activity when heated for 15 minutes at 50° . Destruction of activity was complete after 15 minutes at 60° .

TABLE III

*Effect of Added Fraction P8-7 on Oxygen Uptake (Microliters) of Fraction P8-3A**

Fraction	Time			
	15 min.	30 min.	45 min.	60 min.
P8-3A	2	7	10	14
P8-3A + 0.5 cc. P8-7	12	21	32	43
P8-7	0	0	0	0

* The values shown have been corrected for blanks obtained in the absence of formate. Complete system as for Table II, except that a 10-fold excess of DPN was used to compensate for the rapid destruction of nucleotide.

The specificity of formic dehydrogenase toward coenzyme was demonstrated unequivocally by the failure of the enzyme to reduce TPN^a in the spectrophotometric test. Further, TPN was found not to interfere with the reduction of DPN; therefore it was possible to detect as little as 0.01 mg. of DPN in the presence of 1.00 mg. of TPN.

Enzymic Destruction of DPN—The manometric test for formic dehydrogenase involves a measurement of the rate of oxygen consumption in the presence of formate, DPN, and MB. When the test was applied to the various protein fractions obtained, it was found that some fractions gave a linear rate of oxygen consumption, whereas others showed a decrease in rate of oxygen consumption with time. In the latter case the rate could be restored to the initial level by addition of DPN from a second side arm. These facts are illustrated in Fig. 3, which shows results obtained with the first five fractions of Fraction P8. The conclusion was drawn that an en-

^a Kindly furnished by Dr. Eric Conn of the Department of Biochemistry of the University of Chicago.

zyme was present which destroyed DPN and that this factor was concentrated primarily in the second and third fractions and was absent from the first and fifth.

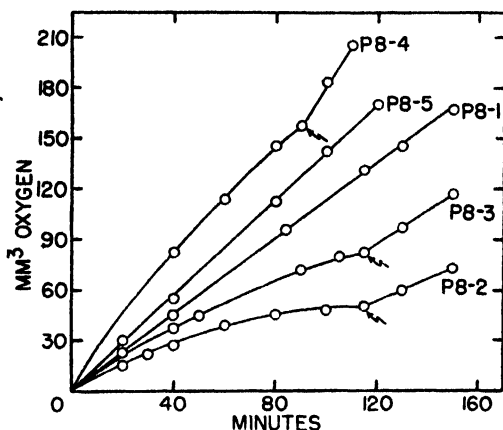


FIG. 3. Destruction of DPN by various enzyme fractions. Complete system as previously described. Additional DPN added from the side arm at points marked by arrows.

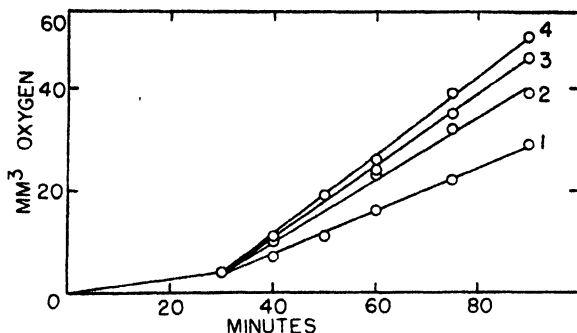


FIG. 4. Relative rates of destruction of DPN and DPNH. Complete system in each flask as in Fig. 2 except for the component indicated as being added from the side arm; 0.001 M ATP in the main compartment of the vessel for Curve 3 only. After 30 minutes incubation the following additions from the side arm were made: Curves 1 and 3, MB; Curve 2, sodium formate; Curve 4, DPN. Fraction P8-3 was used.

The destruction of DPN was inhibited by adenosinetriphosphate and occurred more rapidly with the reduced than with the oxidized form of DPN. These conclusions were drawn from the results of experiments of the type shown in Fig. 4, which were designed to allow a comparison of the relative rates of nucleotide destruction occurring when enzyme was incubated with DPNH, with DPN, and with DPNH and ATP, as shown by Curves 1, 2, and 3, respectively. After 30 minutes of incubation the com-

ponent necessary to complete the system was added, and the resulting rate of oxygen uptake taken as a rough measure of the amount of undestroyed nucleotide remaining (see Table II for the effect of varying DPN concentration).

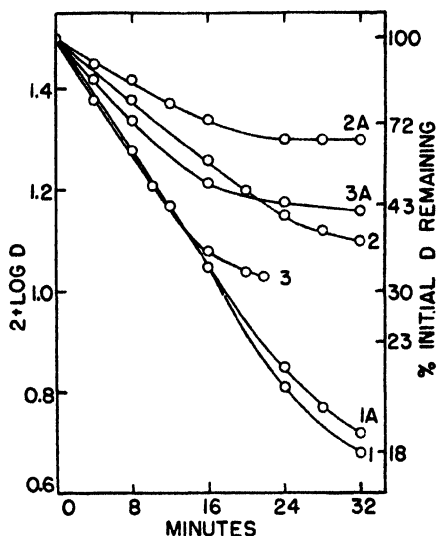


FIG. 5

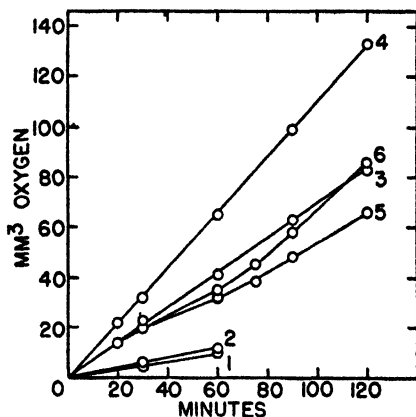


FIG. 6

FIG. 5. Destruction of DPNH in spectrophotometric test. The assay system for the DPNH-oxidizing factor was employed. The DPNH was incubated with the enzyme for 20 minutes prior to the addition of MB, as indicated. Curves 1 and 1A refer to Fraction P8-1, with and without incubation respectively; Curves 2 and 2A, to Fraction P8-2 with and without incubation respectively; Curves 3 and 3A, to Fraction P8-3 with and without incubation respectively. The residual values of $\log D$ obtained in Curves 2A and 3A were not altered by the addition of more enzyme at the end of the test period.

FIG. 6. Oxidation of formic acid in rat liver extracts. Each Warburg vessel contained 2.50 cc. of enzyme, 0.25 cc. of 0.5 M sodium phosphate buffer, pH 7.0, distilled water to a final volume of 3.10 cc., 0.1 cc. of 10 per cent KOH in the center well. Additions were made as follows: Curve 1, none; Curve 2, 0.1 cc. of 0.5 M sodium formate; Curve 3, 0.1 cc. of 0.030 M ATP; Curve 4, 0.1 cc. of 0.030 M ATP and 0.1 cc. of 0.5 M sodium formate; Curve 5, 0.1 cc. of 0.0024 M DPN and, after 60 minutes, 0.1 cc. of 0.030 M ATP; Curve 6, 0.1 cc. of 0.0024 M DPN, 0.1 cc. of 0.5 M sodium formate, and, after 60 minutes, 0.1 cc. of 0.030 M ATP.

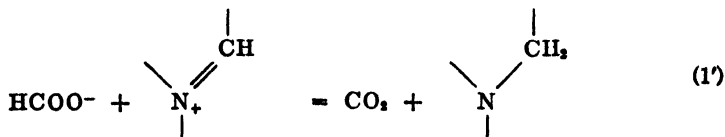
Some indications as to the nature of the structural changes in the DPNH molecule occurring concomitantly with alteration in the enzyme activity of the nucleotide were obtained in spectrophotometric experiments. After incubation of DPNH with various preparations of the oxidizing enzyme, added MB often did not reoxidize the reduced DPNH completely. This is shown in Fig. 5.

It is apparent, however, that incubation of DPNH with enzyme left unchanged the absorption band with a peak at 340 m μ characteristic of the reduced pyridine ring in the DPNH molecule. Since this absorption band was persistent, it appears that inactivation of DPNH did not involve splitting of the nicotinamide-ribose bond. It is possible that the DPNase may have split the nucleotide molecule at the pyrophosphate link in a manner similar to that of the "DPN pyrophosphatase" described by Kornberg and Lindberg (12). Such destruction must be taken into account in the evaluation of the quantitative significance of the assay.

Enzymic Exchange of C¹⁴O₂ in Sodium Formate—From known thermochemical data one may predict that the equilibrium of Equation 1 should lie far to the right.⁴ This is supported by the fact that an oxidation of DPNH by CO₂ cannot be demonstrated spectrophotometrically. A much more sensitive test for the reversibility of the enzymic catalysis is provided by the use of C¹⁴. In the presence of the enzyme system, C¹⁴O₂ should be converted to some extent into HC¹⁴OO⁻. Experiments were carried out to obtain an indication of the order of magnitude of the rate at which CO₂ carbon may be converted to formate carbon in the presence of DPNH and formic dehydrogenase, and also to test the possible influence of adenosinetriphosphate on this reaction.

The following components were incubated for 3 hours at 30°: 5 cc. of enzyme (Fraction P8), 2.5 cc. of 0.5 M sodium formate, 10 cc. of 0.3 M sodium phosphate buffer, pH 8.0, 1.0 cc. of 0.024 M DPN, 0.5 cc. of 0.5 M KH₂PO₄, 0.5 cc. of 0.4 N NaOH containing 0.5 mg. of Na₂C¹⁴O₃. The total CO₂ of the medium was then collected in 4 cc. of 1 N CO₂-free NaOH. The alkali was placed over a sintered glass filter. A stream of CO₂-free air

⁴ The reversible reaction catalyzed by formic dehydrogenase may be given by the following equation, in which the entire molecule of DPN is represented by that portion of the structural formula which undergoes change in the oxidation-reduction.



The equilibrium constant for the above reaction at 30° can be calculated from available thermochemical data. Woods (13) obtained by thermochemical calculation the standard free energy change and the change in heat content at 25° for the reaction catalyzed by hydrogenlyase.



Assuming the above value for ΔH° to be constant over a short temperature range, ΔF° at 30° can be calculated from the relation $d(\Delta F/T)/dT = -\Delta H/T^2$ as -802 calories. Shedlovsky and MacInnes (14) have determined the first ionization constant for carbonic acid. From their equation giving pK₁ as a function of temperature,

was drawn first through the incubation mixture to which 3 cc. of 10 N H_2SO_4 and a drop of capryl alcohol had been previously added and then through the alkali. After 15 minutes the alkali absorbent was removed. Previous tests showed that this procedure sufficed for the quantitative collection of the CO_2 .

An aliquot of the alkali was analyzed for carbonate. To bring the radioactivity to a feasible counting range, a weighed amount of Na_2CO_3 was added. The total dilution was about 1:10,000. Samples of this material were prepared for counting as BaCO_3 by the procedure previously described (16). The results are corrected for dilution by inactive carbonate. All counting data in Table IV are for thick sample counts.

Tank CO_2 was bubbled rapidly through the incubation mixture in order to remove the last traces of remaining C^{14}O_2 . The mixture was centrifuged and the supernatant steam-distilled in a micro-Kjeldahl distillation ap-

the value of pK_1 is calculated as 6.343 for the reaction at 30° .



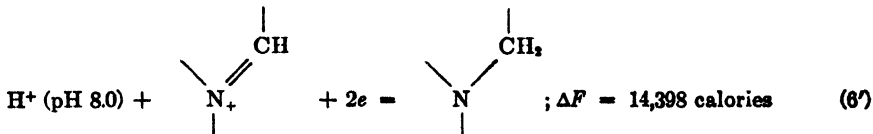
Further, the free energy change is easily calculated for the following reaction at 30° .



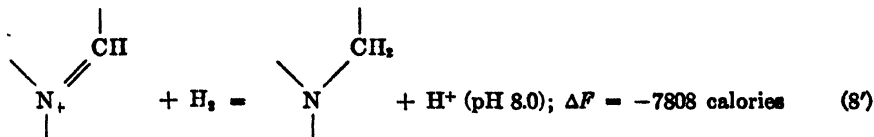
The algebraic summation of Equations 2', 3', and 4' and the corresponding free energy changes for 30° yields the following equation and free energy change at 30° and pH 8.0, with components other than H^+ at unit activity.



Borsook (15) gives the oxidation-reduction potential of DPN at 30° as $E'_0 = -0.072 - 0.03 \text{ pH} \pm 0.0008$ volt. At pH 8.0, $E'_0 = -0.312$ volt. The free energy change at 30° and pH 8.0 with components other than H^+ at unit activity is obtained from the relation $\Delta F = -nFE$, for the reaction



Algebraic summation of Equations 6' and 7' gives Equation 8'.



Algebraic summation of Equations 5' and 8' gives Equation 1', with a free energy change at 30° , $\Delta F^\circ = -6310$ calories. The equilibrium constant for this reaction is therefore $K = 3.5 \times 10^4$.

paratus to recover formic acid. The distillate was made alkaline to methyl red, concentrated by evaporation, transferred to a small aluminum cup, and dried at 120°. The sodium formate thus obtained was counted and then converted to CO₂ by oxidation with HgCl₂, a procedure which does not decarboxylate other saturated acids of the monocarboxylic series possibly present as contaminants (17). In all cases sufficient counts were taken to yield results with a standard deviation of 5 per cent or less.

The above procedure was repeated under identical conditions, except for the addition of ATP, to give a concentration of 0.001 M in the incubation medium. The results are presented in Table IV. Experiments 2 and 2A were carried out concurrently with portions of the same enzyme preparation. Another enzyme preparation was used for both Experiments 3 and 3A, which were also run concurrently. In these experiments aliquots of

TABLE IV
Exchange of C¹⁴O₂ with Formate

Experiment No.	Additions	Carbonate of medium		Activity of formate carbon as BaCO ₃ *	C ¹⁴ of formate C ¹⁴ of medium CO ₂
		c.m.m.	c.p.m.	c.p.m.	per cent × 10 ³
1		1160	24,000,000	20	8.3
2		1500	35,800,000	33	9.4
2A	ATP	1500	36,400,000	30	8.3
3		1320	12,500,000	6.8	5.4
3A	ATP	1240	13,100,000	5.9	4.5

* The values obtained on the dried sodium formate samples agreed with the counts on the BaCO₃ from the formate after appropriate corrections were made for self-absorption.

the incubation mixture were assayed for the total amount of DPNH formed, by determination of the optical density of a diluted sample at 340 mμ before and after the addition of methylene blue. It was necessary to inhibit formic dehydrogenase by 0.001 M HCN during the oxidation of DPNH. The amount of DPNH formed was in the range of 14.0 to 15.9 μM (59 to 66 per cent reduction of DPN), with no noticeable enzymic destruction in any case.

The results of the experiments shown in Table IV indicate that the extent of reduction of carbon dioxide by DPNH is negligibly small. The addition of ATP does not increase the extent of exchange observed.

Oxidation of Formate in Rat Liver and Kidney Extracts—Freshly removed rat livers or kidneys were minced in a glass homogenizer (Potter-Elvehjem type) with an equal volume of distilled water. An additional volume of 0.15 M sodium phosphate buffer, pH 7.0, was added and the

mixture allowed to stand for 30 minutes. After centrifugation for 15 minutes at $5000 \times g$, the hazy supernatant was placed in Visking casings, 1 cm. in diameter, and dialyzed against 0.05 M sodium phosphate buffer, pH 7.0, for 20 hours with constant stirring. The clear supernatant⁵ obtained after centrifugation for 1 hour in a Servall centrifuge at $20,000 \times g$ was used in subsequent experiments. All of the above operations were carried out at temperatures below 6°.

The oxygen consumption of the above extracts was measured manometrically with additions of formate, DPN, ATP, and AMP. It was found that formate was rapidly oxidized in the presence of added ATP or AMP, but not when DPN alone was added. The effect of ATP in stimulating the endogenous oxygen uptake and the oxidation of formic acid in rat liver extract is shown in Fig. 6. This stimulation was also found when AMP was substituted for ATP. Rat kidney extracts gave results similar to those obtained with liver extracts, except that some formic acid oxidation was observed prior to the addition of the stimulating nucleotide.

TABLE V
Carbon Dioxide Formed (Microliters) in Oxidation of Formic Acid

Extract	O ₂ absorbed			CO ₂ released			Ratio of CO ₂ to O ₂ due to formate
	With formate	Without formate	Difference due to formate	With formate	Without formate	Difference due to formate	
Kidney	94	62	32	75	6	69	2.15
Liver	133	83	50	125	19	106	2.12

The production of carbon dioxide during the oxidation of formate was determined by including Warburg vessels containing no alkali in the center well and tipping in 0.1 cc. of 5 N H₂SO₄ at the conclusion of the period of oxidation. Suitable correction for the carbon dioxide present at zero time and all subsequent calculations were made in accordance with established procedure (18). The results (Table V) show that 2 moles of carbon dioxide were produced for each mole of oxygen consumed in accordance with the over-all equation,



⁵ Prolonged centrifugation did not remove the last traces of an initially strong activity capable of oxidizing DPNH. This activity, apparently mainly associated with insoluble particles, was about 90 per cent inhibited by 0.001 M HCN. It interfered with attempts to observe the formation of DPNH spectrophotometrically in experiments designed to test the presence of formic dehydrogenase in these preparations. Liver and kidney preparations contained respectively about 40 and 15 mg. dry weight, per cc., corrected for buffer content.

The presence of 0.001 M HCN inhibited the rate of oxidation of formic acid about 85 per cent and simultaneously reduced the rate of endogenous oxygen consumption by about 25 per cent.

DISCUSSION

The presence in animal tissues of enzymes which catalyze the oxidation of formate indicates that the animal organism may, to some extent, convert formate to carbon dioxide by direct oxidation. The low level of activity observed would not support the conclusion, however, that such oxidations have great metabolic importance. The stimulation of the oxidation of formate by AMP and ATP⁴ appears to differentiate the animal enzymes from the plant enzyme of green peas. In the case of the latter, no direct effect of these nucleotides on formic dehydrogenase was observed, although an indirect effect could undoubtedly be demonstrated with enzyme systems containing the DPN-destroying factor, provided the concentration of DPN were limiting in the assay system chosen. Such indirect effects of ATP and AMP on TPN-specific enzymes of plants have been reported by Conn *et al.* (19).

The resolution of previous formic dehydrogenase preparations into two protein fractions, one of which catalyzes the reduction of DPN, the other, the oxidation of the latter by a dye, follows the previously established pattern of hydrogen transport in analogous systems. The DPNH-oxidizing enzyme was not purified to a sufficient extent to be positively identified as a flavoprotein. It is very probable, however, that such is the case.

Davison (20) has recently reported that formic dehydrogenase is very widely distributed in plant seeds and shows characteristic changes during the life cycle of the plant. Whatever the normal physiological function of this enzyme may be, two properties are worthy of attention. First, its complete specificity for DPN renders it valuable as a specific reagent for detecting this nucleotide. Second, the reaction catalyzed goes to completion; *i.e.*, DPN is, for practical purposes, completely reduced to DPNH. The enzyme is therefore a convenient tool for the quantitative assay of DPN.

The very slow rate at which $C^{14}O_2$ was incorporated into formate in the presence of the enzyme showed that there is no significant direct reduction of CO_2 to formate by DPNH. There is even some question as to whether the positive values obtained might not be caused by minute bacterial contamination. It did not appear worth while to examine this point

⁴ Our results show general agreement with those obtained by Kruhoffer (private communication). He found that the rate of conversion of C^{14} -formate to $C^{14}O_2$ in dialyzed extracts of rat liver was increased 10-fold upon the addition of DPN and ATP.

further, however, since the results corroborate the prediction that the reaction would be too slight to be of physiological importance. It is conceivable, of course, that a significant reduction of CO_2 might occur if the formic dehydrogenase system could be coupled with an energy-yielding reaction. Experiments failed to indicate any such coupling between the formic dehydrogenase system from peas and ATP. Although these negative experiments do not exclude the possibility that such coupling may occur in the intact plant, there appears to be no reason to suppose, at present, that this is the case.

SUMMARY

The enzyme system from green peas catalyzing the reduction of dyes by formate has been shown to consist of two separate factors. One of these, formic dehydrogenase, catalyzes the reduction of DPN by formate; the other catalyzes the oxidation of reduced DPN by methylene blue.

Both enzymes have been partially purified. The properties of the formic dehydrogenase have been studied, particularly with reference to the possible reversibility of the reaction catalyzed. The reduction of CO_2 to formate by reduced DPN was found to occur only to an insignificant extent, and ATP had no effect on the reaction.

Soluble enzymes capable of oxidizing formate to carbon dioxide were shown to be present in rat kidney and liver, but only in small amount. In contrast to the plant enzyme, these animal enzymes were stimulated by ATP and AMP.

We are deeply indebted to Dr. T. R. Hogness for his interest in this problem and for generously placing at our disposal facilities and funds provided by the Rockefeller Foundation. We are also grateful to Dr. T. F. Young and to Dr. C. F. Failey for their advice on the thermodynamic calculations.

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ACETYLCHOLINESTERASE

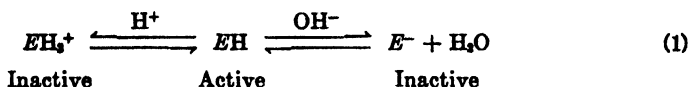
VIII. DISSOCIATION CONSTANTS OF THE ACTIVE GROUPS*

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It was suggested in a preceding paper (1) that the active surface of acetylcholinesterase contains acidic and basic groups. The variation of activity with pH has been interpreted in terms of the acceptance and donation of protons by the active site of the protein according to the formulation



the representation of EH without charge being arbitrary. It is therefore possible to evaluate the dissociation constants of the active group of the enzyme from the data relating the enzymatic activity to the variations of pH. Although pH dependence of various esterases has been reported by a number of investigators (2-4), pH effects upon esterase activity have not been interpreted in terms of acid and basic groups in the active center.

Michaelis and his coworkers interpreted the variation of enzyme activity with pH in terms of the cationic, anionic, and neutral forms of the protein which exist below, above, and at the isoelectric point respectively. The concept of an active center containing only very few functional groups was not developed at that time, and so the activity was therefore associated with the entire protein molecule. The isoelectric point is related to all the basic and acid groups, which are far more numerous than the few functional groups associated with enzyme activity. This constant is therefore a secondary feature which is not directly related to the enzymatic activity. Moreover, in many cases it was not possible to correlate activity with the isoelectric point and therefore the concept was revised by assuming that the activity depended upon the ionization of certain groups in the enzyme molecule (5). This concept was further expanded to include ionization of the substrate (6-9).

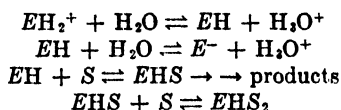
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In this paper appropriate equations will be developed and experiments will be described in which the ionization constants of acetylcholinesterase have been determined. On the basis of the data obtained in this and Paper VII, conclusions as to the possible structure of the reactive groups and the possible mechanism of hydrolysis will be discussed.

It is generally accepted that catalysis proceeds through the formation of an enzyme-substrate complex (10). In many instances the rate is proportional to the concentration of this complex (11). If the activity-substrate relationship has a well defined optimum, *i.e.* is represented by a "bell-shaped curve" (5), then it is generally assumed that the complex reacts with additional substrate molecules and becomes thereby inactive. In Equation 1 it is assumed that only the *EH* form is capable of forming an active complex with the substrate. The choline esters are not altered by changes in pH and, therefore, the substrate is not involved in any equilibria with hydronium ion.

The system is then characterized by the following equilibria



and the corresponding equations, with concentrations as approximations for activities, except for hydronium ion

$$\begin{aligned}\frac{(EH)(H^+)}{(EH_2^+)} &= K_{EH_2^+} \\ \frac{(E^-)(H^+)}{(EH)} &= K_{EH} \\ \frac{(EH)(S)}{(EHS)} &= K_1 \\ \frac{(EHS)(S)}{(EHS_2)} &= K_2\end{aligned}$$

These equations are solved for (EHS) , for the case in which $(S) \gg (E^0)$, where (E^0) is the total enzyme concentration.

If the system may not be properly represented by equilibrium between *EH* and *S*, the equations will not be altered if, as is usual, the reaction can be represented by a steady state in consecutive reactions. In such a case K_1 may not be interpreted as the dissociation constant of *EHS* but rather as $(k_2 + k_3)/k_1$, where k_1 is the bimolecular rate constant for the formation of *EHS*, k_2 the rate constant for the dissociation of *EHS*, and k_3 the rate constant for the further reaction of *EHS* to yield products (5, 12). The reaction velocity is given by $v = k(EHS)$.

$$v = \frac{k(E^0)(S)}{K_1 \left[\frac{(H^+)}{K_{EH_2^+}} + 1 + \frac{K_{EH}}{(H^+)} \right] + (S) + \frac{(S)^2}{K_2}}$$

The solution of $\frac{\partial v}{\partial (S)} = 0$ yields the optimum substrate concentration.

$$(S)_{opt.} = \sqrt{K_1 K_2 \left[\frac{(H^+)}{K_{EH_2^+}} + 1 + \frac{K_{EH}}{(H^+)} \right]} \quad (3)$$

The solution of $\frac{\partial v}{\partial (H^+)} = 0$ yields the optimum hydrogen ion concentration.

$$(H^+)_{opt.} = \sqrt{K_{EH_2^+} K_{EH}} \quad (4)$$

If $K_{EH_2^+} \gg K_{EH}$, the velocity equation can be put in the useful form

$$\frac{v^0}{v} = 1 + \frac{K_1(H^+)}{K_{EH_2^+} \left[K_1 + (S) + \frac{(S)^2}{K_2} \right]} + \frac{K_1 K_{EH}}{\left[K_1 + (S) + \frac{(S)^2}{K_2} \right]} \cdot \frac{1}{(H^+)} \quad (5)$$

where v^0 is the rate at optimum (H^+) . The equation is linear in (H^+) for $(H^+) > (H^+)_{opt.}$ and linear in $1/(H^+)$ for $(H^+) < (H^+)_{opt.}$.

Another useful form is

$$\frac{(S)}{v} = \frac{1}{k(E^0)} \left[K_1 \left(\frac{(H^+)}{K_{EH_2^+}} + 1 + \frac{K_{EH}}{(H^+)} \right) + (S) + \frac{(S)^2}{K_2} \right] \quad (6)$$

This equation is linear in (S) for $(S) \gg (S)^2/K_2$.

To evaluate all the constants, rate determinations must be made as a function of pH at constant (S) and as a function of (S) at constant pH. While the available data (13, 14) describe the phenomena satisfactorily in a semiquantitative way, it is not sufficiently extensive nor precise for the evaluation of the constants. Therefore, the measurements have been repeated with a larger number of experimental points.

Methods

The rate as a function of substrate concentration was measured by the manometric method at a pH of 7.2. At this pH, $(K_{EH})/(H^+)$ of Equation 5 is negligible. Four determinations were made at each concentration. Emphasis was placed upon concentrations below the optimum since these are more important for K_1 , K_2 needing to be known only approximately.

The pH function at $(S) = 4 \times 10^{-3} M$ was determined colorimetrically by using phosphate and carbonate buffers. Non-enzymatic hydrolysis is significant only at pH ≥ 10 . In one set of data indicated in Fig. 2 this

correction was kept small by using larger amounts of enzyme at 1 minute time intervals. These data are therefore given greater weight. pH measurements were made with a glass electrode and were accurate to ± 0.02 unit.

Results

The substrate concentration data are given in Table I. The pH was measured as 7.25. It is not feasible to work at (S) so low that $(S)^2/K_2$ (Equation 6) may be neglected. Therefore, to obtain a linear plot $(S)/v$ is "corrected" to $((S)/v) - (1/k(E^0))((S)^2/K_2)$. The largest correction is less than 9 per cent.

TABLE I

Velocity of Acetylcholine Hydrolysis As Function of Substrate Concentration

v is given as c.mm. of CO_2 developed in 30 minutes. a.d. = average mean deviation.

(S)	Average $(S)/v$ a.d.	$\frac{(S)}{v} - \frac{1}{kE^0} \frac{(S)^2}{K_2}$
3.0×10^{-3}	176 ± 3	161
2.0	118 ± 7	112
1.0	72 ± 6	70
0.75	58 ± 3	57
0.43	51 ± 1	51

K_2 was calculated from the data of Augustinsson and Nachmansohn (13) as 3.2×10^{-2} by Equation 6, with successive approximations of $k(E^0)$, K_1 , and K_2 between high and low substrate concentrations. In the introductory formulation the supercomplex was written as EHS_2 rather than EHS_n , anticipating the result $n = 2$, which was obtained from these data in the usual way.

The y intercept of Fig. 1 yields $1/k(E^0) \left[K_1 \left(\frac{(\text{H}^+)}{K_{\text{EHS}_2^+}} + 1 \right) \right]$, since $K_{\text{EH}}/(\text{H}^+)$ is negligible at this pH and the slope yields $1/k(E^0)$, thus

$$K_1 \left[\frac{(\text{H}^+)}{K_{\text{EHS}_2^+}} + 1 \right] = 4.7 \times 10^{-4} \quad (7)$$

The pH data are plotted in Fig. 2. The acid data are rearranged and plotted in accordance with Equation 5 in Fig. 3. The slope yields

$$\frac{1}{K_{\text{EHS}_2^+}} \left[\frac{K_1}{K_1 + (S) + \frac{(S)^2}{K_2}} \right] = 8.9 \times 10^6 \quad (8)$$

The simultaneous solutions of Equations 7 and 8 yield

$$K_1 = 2.6 \times 10^{-4}$$

$$K_{EH_2^+} = 6.8 \times 10^{-8}; pK_{EH_2^+} = 7.2$$

The alkaline data are plotted in accordance with Equation 5 in Fig. 4.

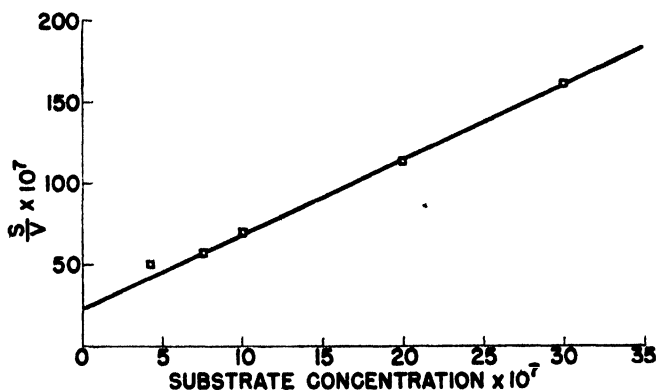


FIG. 1. Relationship between S/v and substrate concentration. The ordinate represents the "corrected" value, $((S)/v) - (1/k(E^o))((S)^2/K_2)$.

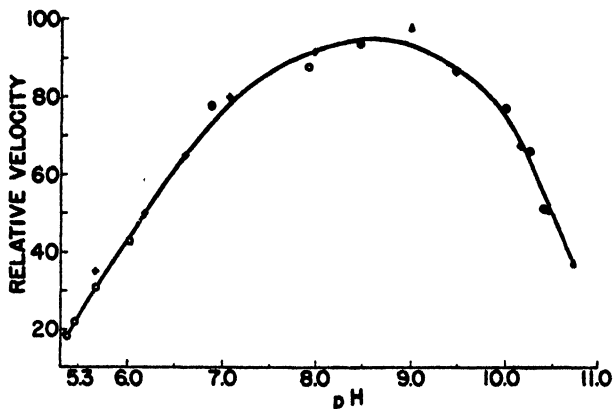
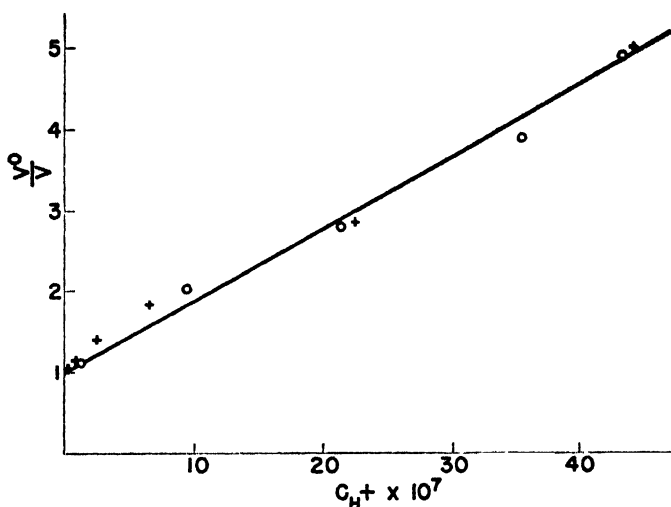
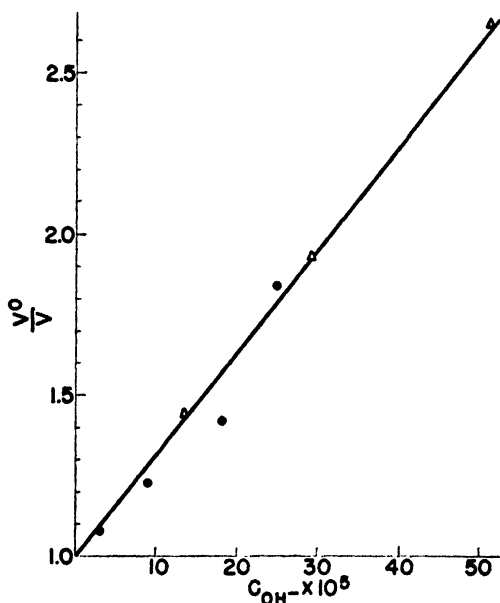


FIG. 2. Velocity of acetylcholine hydrolysis as function of pH

$$K_{EH} = 5.2 \times 10^{-10}; pK_{EH} = 9.3$$

All the constants are for an ionic strength of about 0.15. Beside the assumptions already mentioned, there is implicit in the derivation of Equation 2 the very important assumption that the rate of breakdown of EHS is not affected by (H^+) or (OH^-) , at least within a restricted range of pH. If H^+ (or OH^-) entered the reaction in a rate-controlling step, it would be

FIG. 3. v^0/v as a function of hydrogen ion concentrationFIG. 4. v^0/v as a function of hydroxyl ion concentration

expected to catalyze the reaction, as is the usual case in hydrolysis. The observed change is in the opposite direction, so that any catalysis is less important than the shift in equilibrium. Assuming catalysis by H^+ , we would have:

$$v = k(EHS) [(H^+) + k_2(H_2O)] \quad (9)$$

yielding in place of Equation 6

$$\frac{v^0}{v} = \frac{k_2(\text{H}_2\text{O})}{[\text{H}^+] + k_2(\text{H}_2\text{O})} \cdot \frac{K_1}{K_{\text{EH}_2} + \left[K_1 + (S) + \frac{(S)^2}{K_2} \right]} \cdot \frac{k_2(\text{H}_2\text{O})(\text{H}^+)}{[(\text{H}^+) + k_2(\text{H}_2\text{O})]} \quad (10)$$

Thus v^0/v would "bend over" with increasing (H^+) and a linear relationship would not be obtained. A similar argument applies to OH^- catalysis. Consequently we conclude that neither H^+ nor OH^- enters the reaction in a rate-controlling step.

TABLE II
pH Dependence of Optimum Substrate Concentration

Relative optimum velocity, calculated from Equations 4 and 2, and experimental values observed.

pH	Calculated, ($S_{\text{opt.}}$) $\text{M} \times 10^3$	(EHS)/(E $v_{\text{opt.}}$)	Relative optimum velocity	(S) $\text{M} \times 10^3$	Relative velocity	
					Experiment a	Experiment b
5	35	0.32	39			
5.5	20	0.45	55	3.5	21	17
				12.3	25	42
6	11	0.59	72	3.5	43	35
				12.3	48	36
7	4.5	0.78	96	3.5	94	90
				12.3	81	
8	3.2	0.83	100			
9	3.6	0.82	99			
10	7.2	0.69	84			
11	21	0.43	53			

Equation 3 indicates that the optimum substrate concentration is a function of pH. It increases if the pH is shifted from the optimum. Table II shows the calculated values of $S_{\text{opt.}}$, the fraction of enzyme as complex, and the relative optimum velocity. All optima are flat.

A few experiments have been made to test the shift in optimum substrate concentration. Since the manometric method is restricted to a narrow pH range, the colorimetric method (14) has been used. This latter method determines the hydrolysis by difference of substrate concentrations. The higher the substrate concentrations, the more difficult become precise determinations. This limitation therefore makes difficult an evaluation of the shift in optimum substrate concentration as a function of pH, especially in view of the flat optimum. However, the few observations presented in Table II show a shift in the expected direction.

DISCUSSION

The ionization constants of functional groups in the active surface of the enzyme may serve as another characteristic feature for the description of a particular enzyme or a group of related enzymes.

Whereas usually the Michaelis-Menten dissociation constants are estimated for a given pH, the equations applied show the necessity of taking into account the ionization constants. The value usually reported for K_1 is actually equivalent to $K_1[(H^+)/K_{EH_2^+} + 1 + K_{EH}/(H^+)]$ (see Equation 6). This may result in a considerable difference. In the case under consideration, the value K_1 changes from 4.7×10^{-4} to 2.6×10^{-4} .

The ionization constant may give indications as to possible structures of the active groups involved. The basic group in the acetylcholinesterase, with a $pK_{EH_2^+} = 7.2$, allows the exclusion of carboxylate, phosphate, or guanidine groups. The dissociation constants of most amino groups differ

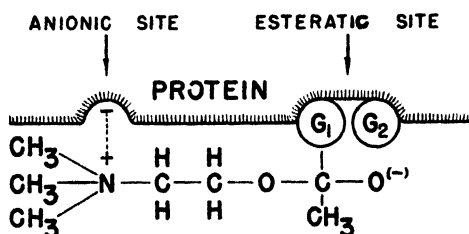


FIG. 5. Hypothetical picture of interaction between the active groups (G_1 and G_2) of acetylcholinesterase and its substrate.

considerably from this value, although in special peptidic combinations such as in tyrosylarginine or phenylalanylarginine (15) the constants are fairly close to the value here determined. Ring nitrogen as in imidazole derivatives has suitable constants; the pK values of imidazole and its methyl derivatives are within the range 7.1 to 7.6 (15).

The second ionization constant, $pK_{EH} = 9.3$, rules out carboxylic and phosphoric acids and guanidinium ions. It is within the range of most biological ammonium groups. Most phenolic hydroxyl groups are out of this range; however, in tyrosine derivatives the ionization constants are near to the above value.

In contrast to non-enzymatic ester hydrolysis, H^+ or OH^- do not appear to be direct reactants in a manner which is rate-determining.

The interpretation of pH dependence of enzyme activity shows that there is a nucleophilic (Group G_1) and an electrophilic (Group G_2) in the active site. The carbonyl group has a marked polarity, the carbon being electrophilic and the oxygen nucleophilic (16). Carbonyl compounds re-

act with nucleophilic reagents, forming addition complexes in which the base forms a bond at the positive carbon. The formation of a bond at the negative carbonyl oxygen greatly increases the electrophilic character of the carbon, thus tending toward an acid catalysis. In the base-catalyzed hydrolysis of esters, the base combines with the electrophilic carbon. The formation of a bond at either of the 2 oxygen atoms (with a proton or with a water molecule via a hydrogen bond) enhances the electrophilic character of the carbon (17, 18).

It appears plausible to assume that the enzyme substrate complex is similar to these reaction intermediates and involves the formation of a bond between the basic group of the enzyme and the positive carbon of the ester. In addition a bond (hydrogen bond) between the acid group of the enzyme and either of the oxygen atoms appears possible. Which oxygen is involved will require further experimentation. In Fig. 5 the combination between the anionic site and the positive ammonium grouping and between the basic group and the carbonyl carbon is illustrated. The possible combination between the acidic group and either oxygen is left open.

SUMMARY

The pH dependence of enzymatic activity is interpreted in terms of acidic and basic groups in the active site. Equations based upon this representation are derived and applied to accurate measurements of pH and substrate dependence of acetylcholinesterase activity.

The following constants were obtained: (1) the apparent dissociation constant of the enzyme-substrate complex $K_1 = 2.6 \times 10^{-4}$; (2) the ionization constant of the conjugate acid of the basic group $pK_{EH_2^+} = 7.2$; (3) the ionization constant of the acid group $pK_{EH} = 9.3$.

These values served as a guide in discussing the possible functional groups in the esteratic site of the enzyme.

The authors wish to express their gratitude to Dr. D. Nachmansohn for his inspiring guidance and advice throughout this work.

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INCORPORATION OF THE CARBONS OF ACETONE, FORMATE, AND CARBONATE INTO ACETOACETATE*

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During the past 15 years it has been demonstrated repeatedly that acetone appears to be metabolized in the animal body. Several workers (1-4) have reported an appreciable rise in the β -hydroxybutyrate and acetoacetate content of various tissues and in the urine of animals which were given acetone. It was not certain from these studies whether acetone was actually converted to β -hydroxybutyrate and acetoacetate or whether it merely stimulated the production of these substances from other sources. The demonstration by Polonovski and Valdiguié (5) that acetate was produced when acetone was incubated in adrenal extract provided more concrete evidence for such a metabolic conversion. The studies of Price and Rittenberg (6) leave no doubt that acetone is actively metabolized in the rat. They administered acetone labeled with C^{14} in the methyl groups to rats and observed that an appreciable amount of the radioactivity appeared in the respiratory CO_2 and in urinary acetyl compounds.

As a result of these studies, the mechanism of the conversion of acetone to a 2-carbon fragment became of interest. Two pathways for this transformation were considered: (a) acetone could be split to C_2 and C_1 fragments, and (b) acetone might be condensed with a C_1 compound to form acetoacetate, which in turn could split into two C_2 fragments. With regard to the latter mechanism, an enzyme has been demonstrated in *Clostridium acetobutylicum* (7) which catalyzes the decarboxylation of acetoacetic acid. A similar catalyst has been claimed to occur in dog blood (8, 9).

Evidence will be presented in this study which demonstrates that the carbons of acetone, formate, and carbon dioxide can be incorporated into acetoacetic acid by rat liver preparations.

Methods and Results

Experiments with Slices—10 gm. of rat liver slices (0.5 mm. thick), prepared with the apparatus of Stadie and Riggs (10), were suspended in

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10 ml. of a medium of the following composition: 40.3 mm of K_2HPO_4 , 8.85 mm of KH_2PO_4 , 76.6 mm of KCl , 0.38 mm of $MgSO_4$, and 10 mm of acetone. The final pH of the medium was adjusted to 7.4. $NaHC^{14}O_3$ and $HC^{14}OOK$ were added to this mixture as indicated.

The reactions were carried out in 125 ml. Warburg vessels which were shaken in a 37° water bath for 2 hours. At the end of the incubation period the reaction was stopped by the addition of 5 ml. of 20 per cent copper sulfate¹ solution. 0.5 mm of sodium acetoacetate (11) was then added as a "carrier." The slices were removed by filtration through cheese-cloth. Freshly prepared calcium hydroxide¹ suspension was added to the mixture to pH 8 to 8.5. The precipitate was centrifuged and the residue was washed by suspension in 20 ml. of water, followed by centrifuging. The supernatant solutions were combined and acidified to pH 2 to 3 with 1 ml. of 2 N HCl and diluted to 150 ml. with water. Free acetone and dissolved carbon dioxide were removed by distillation of the solution *in vacuo* to a volume of 10 ml. This was followed by replenishment of the volume to 150 ml. with water and another concentration *in vacuo* to 10 ml. Solid carbon dioxide was added to displace residual radioactive carbon dioxide. Oxalacetate and oxalosuccinate, which might still be present, were decarboxylated by incubation of this mixture for 2 hours at room temperature in the presence of aluminum sulfate² (12) while a rapid stream of CO_2 -free nitrogen passed through the solution. Dry ice was then added to displace any radioactive CO_2 which may have arisen as a result of the aluminum sulfate treatment. The excess carbon dioxide was removed with nitrogen. Aniline citrate was added to decarboxylate acetoacetate (12). The resulting carbon dioxide was swept out with nitrogen and absorbed in saturated barium hydroxide.

The acetone resulting from the decarboxylation of acetoacetic acid was separated from the residual solution by distillation. The acetone in the distillate was precipitated as the 2,4-dinitrophenylhydrazone in acid solution (13). The radioactivities of the solid samples were determined in a Q-gas counter. Reported values have been corrected for background and self-absorption (14) with the self-absorption curve obtained for $BaCO_3$. The limit of accuracy of the counts has been calculated as the standard error in all cases. All organic compounds were recrystallized to constant radioactivity; their melting points checked with those of the corresponding pure authentic non-isotopic compounds.

The experiments with slices reveal that the carbon of formate and bi-

¹ In studies with homogenates perchloric acid was employed for deproteinization because the use of copper-lime tended to give erratic results.

² In a few experiments aniline citrate (12) at 0° was used for this purpose instead of aluminum sulfate with similar results.

carbonate is incorporated predominantly into the carboxyl group of acetoacetate (Table I). The insertion of the carbon of formate into the carboxyl of acetoacetate was enhanced when liver slices from fasted rats were used. This might be expected from the studies *in vitro* of Annau (15), who observed an increased net production of acetoacetic acid in the livers of fasted rats as compared to that in fed rats.

Homogenate Experiments—Since the slice technique is cumbersome, it was of interest to find a homogenate system in which the C₂ to C₁ condensation could be carried out. The reaction mixture of Potter and Recknagel,³ which in addition to the buffer salts contained malonate and 0.0007 M adenosinetriphosphate, was used for the subsequent homogenate studies.

TABLE I
Incorporation of C¹⁴ from NaHC¹⁴O₃ and HC¹⁴OOK into Acetoacetate in Rat Liver Slices

Experiment No.	Addition, counts per min.	Radioactivity	
		Carbon 1	Carbons 2, 3, and 4
		c.p.m. per mM	c.p.m. per mM
1	9.9 μ M HC ¹⁴ OOK, 1.34×10^6	1,280 \pm 30	
2	9.9 " " 1.34×10^6	23,800 \pm 386	170 \pm 79
3	9.9 " " 1.34×10^6	9,350 \pm 92	370 \pm 58
4	100 " NaHC ¹⁴ O ₃ , 1.06×10^6	2,280 \pm 44	590 \pm 89
5	100 " " 1.06×10^6	12,400 \pm 100	400 \pm 79

In Experiment 1 the rat was fed and in Experiments 2 to 5 the animals were fasted 36 to 48 hours prior to the experiment. Incubated at 37° in air for 2 hours.

The amounts of radioactive indicators and the enzyme concentrations employed are noted in Tables I to VI. The amount of acetoacetate carrier added and the subsequent method of degradation were the same as those described for the experiments with slices.

As in the case of the slices, the homogenates incorporated a significant amount of the carbon of formate and of carbonate into the carboxyl group of acetoacetate. Even when NaHC¹⁴O₃ of very high specific activity was employed and a particularly large incorporation of C¹⁴ into the carboxyl group of acetoacetate resulted, the activity of the acetone portion of the molecule was very small (Table II). It is uncertain whether formate is incorporated into the carboxyl group of acetoacetic acid as such or whether it has to be converted to carbon dioxide prior to being thus utilized. Under the conditions of our experiments enough carbon dioxide is formed from

³ We wish to thank Dr. Van R. Potter and Dr. R. O. Recknagel for providing this information before publication.

TABLE II
Incorporation of C^{14} from $NaHC^{14}O_3$ and $HC^{14}OOK$ into Acetoacetate by
Homogenates of Rat Organs

Experi- ment No.	Addition, counts per min.	Malonate	Radioactivity	
			Carbon 1	Carbons 2, 3, and 4
		M	c.p.m. per mm	c.p.m. per mm
1	9.9 μM $HC^{14}OOK$, 1.34×10^6	0.0028	710 \pm 25	0
2	9.9 " " 1.34×10^6	0.0028	3,400 \pm 36	0
3*	9.9 " " 1.34×10^6	0.0071	7,420 \pm 67	465 \pm 14
4	100 " $NaHC^{14}O_3$, 1.06×10^6	0.0028	590 \pm 20	0
5	5.4 " " 8.17×10^6	0.0071	47,900 \pm 216	140 \pm 24
6	5.4 " " 8.17×10^6	0.0071	50,300 \pm 302	
7	5.4 " " 8.17×10^6	0.0071	59,100 \pm 296	
8A†	5.4 " " 8.17×10^6	0.0071	0	
8B	5.4 " " 8.17×10^6	0.0071	24,500 \pm 204	
9A††	5.4 " " 8.17×10^6	0.0071	0	
9B†	5.4 " " 8.17×10^6	0.0071	29,300 \pm 242	
10§	5.4 " " 8.17×10^6	0.0071	390 \pm 54	
11	5.4 " " 8.17×10^6	0.0071	1,840 \pm 28	
12¶	55.4 " " 8.7×10^6	0.0071	1,300,000	

9.6 ml. of 20 per cent homogenate in isotonic KCl were added to all the flasks except in Experiment 1 in which 4.8 ml. of 10 per cent homogenate were used. Rat liver was used in all the experiments except in Experiments 10 and 11. Final volume 17 ml. Incubated at 37° in air for 2 hours except in Experiments 9 to 12.

* 70 per cent of the radioactivity of the added formate was recovered in the respiratory CO_2 after stopping the reaction with $HClO_4$. The respiratory CO_2 had a specific activity of 1.57×10^7 c.p.m. per mm.

† Homogenate heated in a boiling water bath for 15 minutes.

‡ Incubated for 15 minutes.

§ Heart homogenate. 20 μM of acetoacetate present during the 15 minute incubation period.

|| Kidney homogenate. 20 μM of acetoacetate present during the 15 minute incubation period.

¶ This value represents the actual specific activity of the carboxyl group of acetoacetate and was derived as follows: after the addition of $HClO_4$ at the end of the 15 minute incubation period, the resulting mixture was divided into two portions. Acetoacetate carrier was added to an aliquot and the specific activity of the carboxyl group was determined as usual (4900 c.p.m. per mm). The acetoacetate concentration in the filtrates with and without added carrier acetoacetate was determined (18) and was found to be 50.5×10^{-3} mm per ml. and 0.19×10^{-3} mm per ml. respectively. Then the specific activity of $-COOH$ (plus carrier) \times (acetoacetate (carrier present))/(acetoacetate (carrier absent)) = $4900 \times ((50.5 \times 10^{-3})/(0.19 \times 10^{-3})) = 1.3 \times 10^6$ c.p.m. per mm = actual specific activity of $-COOH$.

formate to account for the insertion of the carbon of formate into acetoacetate via carbonate (Experiment 3, Table II). Fixation of carbon dioxide

into the carboxyl group of acetoacetate was observed with kidney homogenates but it is not certain whether this reaction occurs to any significant extent in heart muscle preparations (Table II).

Incorporation of Acetone—Carbonyl-labeled acetone was prepared by pyrolysis of carboxyl-labeled barium acetate (14). The degradation methods employed in these experiments were identical with those described except that during the course of the removal of the residual, free isotopic acetone *in vacuo* large quantities of non-isotopic acetone (2.7 moles) were added. This treatment was adequate to prevent significant contamination of acetone obtained from the acetoacetate degradation by the labeled compound initially added.

TABLE III
*Incorporation of C¹⁴ from Carbonyl-Labeled Acetone into Acetoacetate
in Rat Liver Homogenates*

Experiment No.	Malonate	Radioactivity		
		Carbon 1	Carbons 2, 3, and 4	Respiratory CO ₂ *
		c.p.m. per mM	c.p.m. per mM	c.p.m. per mM
1	0.0028	2110 ± 37	9,800 ± 98	1480 ± 26
2	0.0071	790 ± 17	10,000 ± 84	875 ± 33

33 μ M of carbonyl-labeled acetone, 1.11×10^6 c.p.m., and 9.6 ml. of 20 per cent rat liver homogenate per flask. Final volume 17 ml. Incubated at 37° in air for 2 hours.

* The specific activity of the respiratory CO₂ cannot be compared directly with that in the acetoacetate since no "carrier" was present during the incubation period.

The results of this study (Table III) indicate that the carbons of added acetone are incorporated into the acetone portion of acetoacetate. In the case in which only 0.0028 M malonate was used to inhibit the tricarboxylic acid cycle, a considerable portion of the carbonyl carbon of the added acetone was found in the carboxyl groups of the formed acetoacetate. When the malonate concentration was increased to 0.0071 M, the incorporation into the carboxyl groups was decreased while the radioactivity of the acetone portion remained constant. A small amount of the activity was also found in the respiratory CO₂. This conversion of acetone to carbon dioxide may be due to incomplete blocking of the Krebs cycle by malonate or to the exchange of the carboxyl of formed acetoacetate with the surrounding medium.

While the incorporation of the carbonyl carbon of acetone into acetoacetate was demonstrated (Table III), the insertion of C¹⁴-carbonate into the carboxyl group was not enhanced by the addition of acetone to the homogenate or washed residue enzyme systems (Table IV). It was con-

sidered that the lack of effect of acetone here might be caused by the endogenous production of an adequate amount of acetoacetate by these systems. A requirement for acetone could, however, not be shown even in the presence of atoxyl (Table IV), which Jowett and Quastel (16) found to decrease endogenous acetoacetate formation. Furthermore, the addition of acetoacetate to incubating liver homogenates did not lead to an increased fixation of CO_2 into the carboxyl group. However, the incorporation of carbon dioxide into the carboxyl group of acetoacetate was enhanced by the addition of pyruvate (which yields acetoacetate under these experimental conditions ((17); see also foot-note 3) to the incubation

TABLE IV
Effect of Acetone on Incorporation of C^{14} from $\text{NaHC}^{14}\text{O}_3$ into Carboxyl Group of Acetoacetate

Experiment No.	Enzyme*	Addition	Radioactivity
			<i>c.p.m. per mm</i>
1	Homogenate	1 μM acetone	50,400 \pm 302
		No acetone	59,200 \pm 296
2	Washed residue	1 μM acetone	27,800 \pm 158
		1 " " + 1 μM α -ketoglutarate	9,200 \pm 75
		1 " α -ketoglutarate	15,300 \pm 125
3	" "	1 " acetone	29,700 \pm 211
		No acetone	33,500 \pm 261
4	" "	1 μM acetone, 0.01 M atoxyl†	11,500 \pm 151
		No acetone, 0.01 M atoxyl	9,200 \pm 143

5.4 μM of $\text{NaHC}^{14}\text{O}_3$ at 8.17×10^4 c.p.m. were added to all the flasks. Incubated at 37° in air for 2 hours; 0.0071 M malonate present.

* From rat liver tissue.

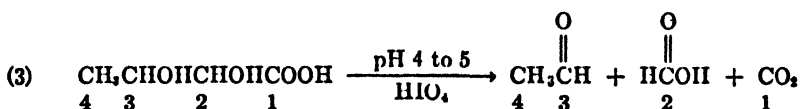
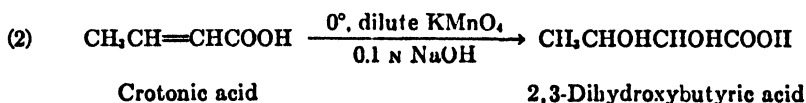
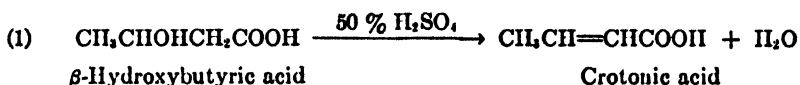
† Sodium arsanilate.

mixture (Experiment 8, Table VI). It is not clear whether this effect is due to the production of a nascent form of acetoacetate from pyruvate or to the generation of energy utilizable in the condensation reaction. The chemical methods employed may not differentiate between acetoacetate and possible derivatives of this compound which may be present. The actual endogenous acetoacetic acid formation was determined⁴ by Lehninger's unpublished modification of the method of Greenberg and Lester (18) in one experiment in which a washed liver homogenate was used as the enzyme. On the basis of this assay and the specific activity of added $\text{NaHC}^{14}\text{O}_3$, it was calculated that on a molar basis 0.83 per cent of the

⁴ We are indebted to Miss Gladys Feldott who conducted the dietary experiments and performed the acetoacetate determinations.

carboxyl groups and of the acetoacetate present had been derived from bicarbonate (Experiment 12, Table II).

Experiments with β -Hydroxybutyrate—To eliminate the element of uncertainty regarding the specificity of the direct degradation of acetoacetate in the deproteinized medium it seemed desirable to use a more specific method of degradation. Since β -hydroxybutyric acid and acetoacetic acid are interconvertible in the liver (19), a procedure, based on the isolation of β -hydroxybutyric acid and its subsequent degradation, was developed. At the end of the incubation period, 180 mg. of carrier sodium DL- β -hydroxybutyrate were added to the reaction mixture. After deproteinization with copper-lime, the filtrate was acidified with 1 ml. of 7 N sulfuric acid. Volatile fatty acids were removed by steam distillation. The residual solution was neutralized to pH 5 to 6 with sodium hydroxide. 1 gm. of sodium bisulfite per 15 ml. of solution was added to combine with



keto acids. 7 gm. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.45 ml. of 7 N H_2SO_4 were added and the solution was continuously extracted with ether for 96 hours. The extract was evaporated to dryness *in vacuo*. The β -hydroxybutyric acid in the residue was converted to crotonic acid by refluxing with 50 per cent H_2SO_4 according to the method of Darmstädter (20). The crotonic acid in the distillate was extracted into ether. The ether layer (400 to 500 ml.) was washed two to three times with 5 ml. of water and dried with anhydrous Na_2SO_4 . The dried ether extract was evaporated to 10 to 15 ml. and an ethereal solution containing 0.8 gm. of *p*-dimethylaminophenylcarbodiimide was added. The solution was refluxed for several hours and left to stand at room temperature for a few days until the precipitation of the ureide of crotonic acid was complete (21, 22). The ureide (m.p. 151–152°) was recrystallized to constant radioactivity. It was hydrolyzed with 85 per cent H_3PO_4 according to Zetzsche and Röttger (21). The acid solution was diluted with 2 volumes of water and extracted with ether; the solvent was washed with a few small volumes of water and the crotonic acid

was extracted from the solvent with 15 ml. of 0.1 N NaOH. The aqueous solution was chilled to 0° in an ice bath and the crotonate was oxidized to dihydroxybutyrate (23) by the dropwise addition of dilute KMnO_4 to the first tinge of pink. The excess KMnO_4 was destroyed *immediately* with a small quantity of sodium hydrosulfite and MnO_2 was removed by centrifugation. The supernatant was acidified with 0.3 ml. of glacial acetic acid and evaporated *in vacuo* to 10 ml. The solution was adjusted to pH 4 to 5 with 0.1 N NaOH. The 2,3-dihydroxybutyric acid was degraded to CO_2 , formic acid, and acetaldehyde by the addition of 5 ml. of 0.1 M periodic acid, and the products were collected separately. The carbon dioxide was precipitated as BaCO_3 , acetaldehyde was converted to the 2,4-dinitrophenylhydrazone, and formic acid was decomposed to carbon dioxide

TABLE V

Incorporation of HC^{14}OOH and $\text{NaHC}^{14}\text{O}_3$ into β -Hydroxybutyrate by Rat Liver

Experiment No.	Addition, counts per min.	Radioactivity			
		Ureide carbon of crotonic acid	Carbon 1	Carbon 2	Carbons 3 and 4
		<i>c.p.m. per mM</i>	<i>c.p.m. per mM</i>	<i>c.p.m. per mM</i>	<i>c.p.m. per mM</i>
1	19.8 μM HC^{14}OOK , 2.68×10^6	1210 \pm 29	1210 \pm 20	0	0
2	5.4 " $\text{NaHC}^{14}\text{O}_3$, 8.17×10^6	1400 \pm 36	1225 \pm 17	0	0

In Experiment 1, slices incubated aerobically for 1 hour; 180 mg. of sodium DL- β -hydroxybutyrate then added and incubated under N_2 for 45 minutes.

In Experiment 2 with homogenate, 0.0071 M malonate present. Homogenate incubated in air for 30 minutes. Then 75 μM of α -ketoglutarate were added and incubated under N_2 for 30 minutes. Final volume 17 ml. The oxidation of α -ketoglutarate is coupled with the reduction of acetoacetate (36).

with mercuric chloride (24). Pilot experiments with non-isotopic crotonic acid revealed that a 90 per cent recovery of the split-products (Equation 3) could be obtained.

The data obtained with this procedure revealed that the carbons of formate and carbon dioxide were incorporated exclusively into the carboxyl group of β -hydroxybutyric acid (Table V). It should be pointed out that no insertion of the carbon of formate or carbon dioxide into β -hydroxybutyrate could be demonstrated unless the additional anaerobic reductive steps described in Table V were used. This may indicate that very little or no β -hydroxybutyrate is formed under our aerobic experimental conditions.

Effect of Biotin—Biotin has been implicated in a number of carbon dioxide fixation reactions (25–29). A study was made to ascertain whether a similar situation prevailed in the acetoacetate reaction.

Weanling Sprague-Dawley rats were grown for 10 weeks on a purified diet⁵ containing raw egg white without added biotin (27).⁴ An equal number of rats was fed a similar diet but with added biotin and without raw egg white, and their food intake was restricted to that of a biotin-deficient animal. The animals fed the egg white diet developed typical symptoms of biotin avitaminosis.

TABLE VI

Effect of Biotin Deficiency on Incorporation of C¹⁴ from NaHC¹⁴O₃ into Carboxyl Group of Acetoacetate

Experiment No.	Control ration fed	Radioactivity	
		Control animal	Deficient animal
		c.p.m. per mg	c.p.m. per mg
1	+ dietary biotin, pair-fed	44,200 ± 398	9,600 ± 58
2	+ " " "	30,200 ± 151	14,000 ± 140
3	+ " " "	20,600 ± 144	5,800 ± 41
4	+ " " "	48,000 ± 215	15,300 ± 127
	Stock ration <i>ad libitum</i>	50,300 ± 302	
5*	+ dietary biotin, pair-fed	22,400 ± 163	735 ± 18
6	Biotin-deficient + injected biotin,† pair-fed	52,500 ± 210	8,500 ± 94
7	Biotin-deficient + injected biotin,† pair-fed	71,000 ± 284	6,550 ± 85
8A	Stock ration <i>ad libitum</i>	8,750 ± 146	935 ± 66
8B†	" " " "	25,300 ± 228	3,150 ± 44

9.6 ml. of 20 per cent rat liver homogenate (variations of tissue dry weights between different flasks did not exceed ±5 per cent); final volume 17 ml., 0.0071 M malonate present. Homogenates incubated in air at 37° for 120 minutes, except Experiments 5 and 8. 5.4 μM of NaHC¹⁴O₃ containing 8.17×10^6 c.p.m. added except in Experiment 8 in which 2.7 μM of NaHC¹⁴O₃ containing 4.08×10^6 c.p.m. were used.

* 20 μM of acetoacetate present during the 15 minute incubation period.

† 50 γ of biotin injected intraperitoneally daily for 4 days.

‡ 60 μM of sodium pyruvate present during the 15 minute incubation period.

A smaller incorporation of C¹⁴ from carbonate into the carboxyl group of acetoacetate was obtained in every case when liver homogenates from biotin-deficient rats were used in comparison with enzyme preparations from either deficient animals injected with biotin or from control rats (Table VI). These differences were particularly apparent when the incubation time was reduced from 120 to 15 minutes (Experiments 5 and 8, Table VI).

⁵ The vitamin levels quoted previously (27, 28) were for 10 gm. of ration rather than 100 gm., as stated.

It appears from this data that the state of biotin nutriture influences this new carbon dioxide fixation reaction too.

DISCUSSION

The equilibrium of the reaction



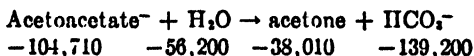
is so far in the direction of decarboxylation that it would be impossible to explain the incorporation of C^{14} into acetoacetate found here merely by an equilibrium exchange of the reactants and products.⁶ Therefore, it is likely that an energy-coupling process is a part of the mechanism of this reaction.

The failure of Swendseid *et al.* (30) to demonstrate any incorporation of C^{18}O_2 into ketone bodies in the whole rat may have been due to the excessive dilution of the isotope in the body, the rapid loss of the carbon in the respiratory carbon dioxide, and the competition of more rapid carbon dioxide fixing reactions.

The $\text{C}_2 + \text{C}_1$ condensation leading to acetoacetate could provide a pathway by which carbon dioxide could be inserted into the carboxyl group of "acetate." This mechanism may account for Schubert and Armstrong's (31) observation that the carboxyl groups and presumably the other odd carbons of fatty acids were tagged as a result of the administration of C^{14} -carbonate to rats. It has already been pointed out by others (32, 33) that such a labeling of the fatty acids could not occur by the metabolism of CO_2 via the tricarboxylic acid cycle.

The finding by Coon and Gurin (34) that the γ -carbon of leucine appears in the carbonyl group of acetoacetate could be explained by the reaction studied here, particularly since their evidence points to the formation of an acetone-like fraction from the isopropyl group of leucine.⁷ In this connection it has been observed by Valdiguié and Séguélas (35) that acetone was produced when cholesterol and coprosterol were incubated in liver ex-

⁶ The equilibrium constant was calculated from the free energy of formation of the reactants and products at 37.6°. The values of the free energies of formation are for 1 molal activity, except for water; in the latter case they are for pure water. These values are from unpublished work of Professor Henry Borsook, California Institute of Technology. We wish to thank Professor Borsook for providing these data.



$\Delta F = -16,300$ calories; $K = ((\text{acetone}) (\text{HCO}_3^-))/(\text{acetoacetate}^-) (\text{H}_2\text{O})) = 3 \times 10^{11}$.

⁷ In a personal communication Dr. M. J. Coon and Dr. S. Gurin have informed us that they too have observed the incorporation of the carbon of carbon dioxide into the carboxyl group of acetoacetate.

tracts. These workers are of the opinion that this acetone is derived from the isopropyl portion of the sterol side chain.

SUMMARY

The carbons of formate and carbonate were incorporated into the carboxyl group of acetoacetate by rat liver slices and homogenates. This finding was confirmed by the isolation and degradation of β -hydroxybutyric acid, the reduction product of acetoacetate. Additions of pyruvate to the reaction mixture increased the fixation of carbon dioxide into acetoacetate, but acetone and α -ketoglutarate did not enhance this incorporation of CO_2 .

Formate was rapidly oxidized to carbon dioxide by rat liver preparations and possibly was incorporated into acetoacetate only after this conversion.

Carbon dioxide was also fixed into the carboxyl group of acetoacetate by kidney homogenates and perhaps in the presence of heart enzyme preparations.

In rat liver homogenates the radioactivity of carbonyl-labeled acetone was incorporated predominantly into the acetone portion of acetoacetate. When low concentrations of malonate were used to inhibit the tricarboxylic acid cycle, a considerable portion of the carbonyl carbon of added acetone was also found in the carboxyl group of acetoacetate. A small portion of the C^{14} from added acetone was also detected in the respiratory carbon dioxide.

The fixation of carbon dioxide into acetoacetate by liver homogenates from biotin-deficient rats was much less than by homogenates from rats receiving biotin.

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STUDIES ON THE METABOLISM OF THIOUREA

II. THE METABOLIC FATE OF THIOUREA IN THE THYROID GLAND*

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Thiourea is the parent compound of a group of compounds known to have goitrogenic activity. These substances prevent the formation of organically bound iodine from iodide ion (1-6), but the mechanism of this action is unknown. Paper I of this series (7) showed that when thiourea labeled with radioactive sulfur was administered to rats the concentration of radioactivity in the thyroid gland 24 hours later was 30 times as high as in any other tissue. The chemical nature of the radioactive sulfur in the gland, however, was not determined at that time. The experiments described in this report attempt to show that the sulfur of thiourea was oxidized to sulfate ion in the thyroid gland of the rat.

EXPERIMENTAL

The activity in the rat thyroid following intraperitoneal injection of S³⁵-thiourea was shown to be freely dialyzable by the following experiment. Animals were sacrificed 24 hours after administration of the radioactive material. The thyroid gland was ground in a glass homogenizer with 1 ml. of distilled water which had been boiled to remove dissolved oxygen. The homogenate was transferred to a Visking sausage casing which contained a glass-enclosed magnetic stirring bar. The dialyzing bag was then placed in an Erlenmeyer flask containing 35 ml. of cold, distilled, boiled water. The air above the fluid was flushed out with nitrogen and the flask corked. The preparation was placed in a cold room (5°) and the bag constantly rotated with a magnetic induction stirrer. The dialysate was changed several times a day and each sample was assayed individually for radioactivity as previously described (7). The results are shown in Fig. 1, in which the integrated radioactivity is plotted against time. Only 0.25 per cent of the total radioactivity in the gland remained undialyzed after 100 hours.

The dialyzable radioactivity in the thyroid gland following administra-

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tion of radioactive thiourea might have been in the form of unchanged thiourea, sulfide ion, sulfate ion, or free sulfur. The form of the radioactive material was studied by preparing a 24 hour dialysate of the gland essentially as described above. It was then made up to volume and aliquots taken for study.

One aliquot was extracted twice with carbon disulfide. The carbon disulfide was evaporated with an air stream and the residue was assayed for radioactivity.

To two other aliquots were added both 5 mg. of sodium sulfide and 15 mg. of sodium sulfate to serve as carriers. 250 mg. of barium chloride were added to one of these aliquots and 50 mg. of cadmium chloride in

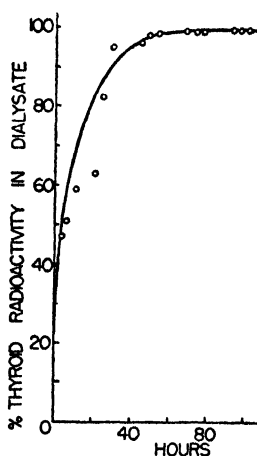


FIG. 1. Dialysis of radioactive material in the thyroid gland after injection of S^{35} -thiourea.

0.1 N hydrochloric acid were added to the other. The resulting precipitates were collected, placed on copper disks, and counted for radioactivity (7). The data are presented in Table I. These results show that most of the activity was precipitated with barium sulfate. Very little activity was precipitated with cadmium sulfide and very little was extracted with carbon disulfide.

Barium sulfate is known to adsorb appreciable amounts of unrelated substances, and the amount of radioactive material in the thyroid gland in these experiments was small (of the order of 10^{-6} mg.). Therefore, a fractional precipitation was carried out to prove further that the radioactive material in the gland was sulfate ion.

A dialysate was prepared as before and an aliquot taken for determination of total radioactivity. Two other equal aliquots were taken for

fractional precipitation of barium sulfate. To one were added 24.4 mg. of sodium sulfate (equivalent to 40.9 mg. of barium sulfate), and to the other 91.5 mg. of sodium sulfate (equivalent to 150 mg. of barium sulfate). To each of these aliquots were added 22.3 mg. of barium chloride to precipitate exactly 25 mg. of barium sulfate. The precipitates were collected, placed on copper disks, and counted. Table II shows that in both cases the percentage of the radioactivity precipitated from the aliquot was the same as the percentage of the sulfate precipitated. If the radioactivity had been adsorbed on the barium sulfate precipitate, the same amount of activity should have come down in both experiments, because the size

TABLE I
Partition of Radioactive Material in Dialysate of Thyroid of Rat Following Administration of S^{35} -Thiourea

Fractionation procedure	Activity
	<i>per cent</i>
Precipitation with cadmium sulfide	6.61
" " barium sulfate	97.0
Extraction with carbon disulfide	7.71

TABLE II
Fractional Precipitation with Barium Sulfate of Radioactive Material from Dialysate

	Aliquot A	Aliquot B
Total SO_4 present as $BaSO_4$, mg.....	40.9	150
$BaSO_4$ precipitated, mg.....	25	25
Total radioactivity, c.p.s.....	40.7	40.7
Radioactivity precipitated, c.p.s.....	23.8	7.24
SO_4 precipitated as $BaSO_4$, %.....	61	16.7
Radioactivity precipitated, %.....	58.8	17.8

of the two precipitates was the same (25 mg.) and adsorption depends primarily on the surface area of the precipitate. Since the percentage of the radioactivity precipitated was equal to the percentage of sulfate ion precipitated, it seems reasonable to conclude that the radioactivity was actually precipitated as barium sulfate and was, therefore, in the form of sulfate ion (or a closely related oxy acid of sulfur) in the gland.

The radioactive material in the thyroid gland was further studied by filter paper partition chromatography, thus providing additional proof of its identity, based on a different type of procedure.

Thyroid glands of rats injected with radioactive thiourea 24 hours previously were homogenized with 1 ml. of distilled water. The material

was centrifuged to remove gross particles of insoluble matter and the supernatant placed on 60 × 2 cm. strips of Whatman No. 1 filter paper. Chromatograms were developed and recorded on x-ray film as described previously (7). As controls, aqueous solutions of radioactive sulfuric acid and thiourea were chromatographed simultaneously. The R_f values (ratio of the migration of the substance analyzed to the migration of the solvent front) are shown in Table III. The R_f values for the radioactive substance in the gland corresponded closely to those of sulfuric acid and differed unmistakably from those for thiourea.

As a further test, a mixture of the extract of the gland and radioactive sulfuric acid was partitioned on filter paper in several solvents. In each case only a single band was found, providing further evidence that the radioactive material in the gland was sulfate ion.

TABLE III

Comparison of Chromatographic Data for Sulfate Ion, Thiourea, and Radioactive Substance of Rat Thyroid Following Administration of S^{35} -Thiourea

Solvent system	R_f value		
	Unknown	Sulfuric acid	Thiourea
Ethyl methyl ketone-1 N hydrochloric acid	0.05	0.06	0.56
“ “ ketone-water.	0	0	0.60
n-Butanol-1 N ammonium hydroxide.	0	0	0.46
n-Butanol-1 N acetic acid	0.17	0.16	0.59
n-Butanol-water	0	0	0.45
Collidine-water	0.08	0.07	0.77

As further proof that there was no thiourea in the thyroid, the thyroid of a rat injected with radioactive thiourea 24 hours previously was homogenized with 0.1 ml. of water and 100 mg. of non-radioactive thiourea. This homogenate was then extracted twice with 5 ml. portions of ethanol. The combined extracts were evaporated to dryness and the residue taken up in 2 ml. of boiling butanol and filtered. On cooling, crystals of thiourea appeared. These were dried, found to melt at 172° (reported 172–174°), and a portion assayed for radioactivity. There was no radioactivity in the crystals. All the activity was in the ethanol-insoluble portion of the gland.

DISCUSSION

It was shown previously (7) that when 1 mg. of sulfur-labeled thiourea was administered to a rat about 10 per cent of the sulfur appeared in the urine as sulfate ion. It was also shown that 24 hours after injection the concentration of radioactive sulfur in the thyroid was 30 times as great

as in any other tissue in the body. The work presented here indicates that the radioactive sulfur in the thyroid was in the form of sulfate ion. When, however, sulfate ion was administered to an animal, it was uniformly distributed (7). Therefore, a portion of the sulfur of thiourea must have been oxidized to sulfate in the thyroid gland. This high concentration of radioactive sulfur in the thyroid might be explained as follows: Cells are very permeable to thiourea but only moderately permeable to sulfate ions (8). Therefore, in the organ in which appreciable oxidation occurred (the thyroid), the radioactive sulfur left the cells more slowly than it entered them. In the other cells of the body, however, it did not undergo appreciable oxidation to sulfate and left at the same rate at which it entered.

The thyroid gland takes up circulating iodide and converts it into thyroxine. These two functions are apparently separate and the second is prevented by thiourea and related compounds (1-6). The use of iodide ion in the synthesis of thyroxine must involve oxidation, since the iodine in thyroxine is in a higher valence state. The mechanism for this oxidation is not definitely known. Some workers claim that peroxidase is present in the thyroid gland and that the properties of thyroid extracts are the same as those of peroxidase (9-11), but other workers deny the existence of peroxidase in the gland (12). It has been shown that peroxidase is inhibited by thiourea *in vitro* (13), and it has been postulated that thiourea inhibits the oxidation of iodide ion *in vivo*. Some workers have claimed that there is no decrease in total oxygen uptake by the gland *in vitro* when thiourea is present (14-16) and that there is no decrease in cytochrome oxidase (17, 18) or succinic dehydrogenase activity (18, 19). Others claim a decrease in cytochrome oxidase activity (20-23). A decrease in peroxidase activity has also been described (9-11).

Two types of interference with this oxidative mechanism can be postulated. Thiourea might inactivate an enzyme or it might compete with the enzyme substrate. The observation that thiourea was oxidized in the thyroid gland more rapidly than elsewhere would make enzyme inactivation unlikely. It would require the postulation of two unique oxidizing systems in the thyroid, one of which oxidized iodide ion and the other of which cannot oxidize iodide ion but can oxidize thiourea after the first system has been inactivated.

Competition with the substrate is fully compatible with the known facts. Iodine will oxidize thiourea *in vitro* (24) and it has been shown above that thiourea is oxidized by the thyroid gland *in vivo*. When 1 mg. of thiourea was administered to a rat, only 100 γ were oxidized (7). The blood flow in the thyroid is known to be about 0.05 ml. per minute (25). Assuming that the concentration of thiourea in the blood decreased exponentially,

one can calculate that at least 300 γ of thiourea were presented to the thyroid in this time. Therefore, the oxidizing capacity of the gland is apparently limited and can be saturated. This figure of 100 γ of thiourea oxidized in 24 hours can be compared with the value of 10 γ of iodine fixed by the thyroid of a rat after the administration of 0.5 mg. of potassium iodide (26).

If the action of thiourea is due to its preferential oxidation in the thyroid, a new method now is available for the study of this oxidation. The rate of formation of organically bound iodine may well involve a biological mechanism in addition to simple oxidation, whereas the oxidation of thiourea, measured as described in this paper, does not.

SUMMARY

By the use of tracer techniques it has been shown that thiourea, administered to a rat, is oxidized to sulfate in the thyroid gland.

It has been suggested that this compound prevents the formation of organically bound iodine by saturating the mechanism of the thyroid gland, which, under normal conditions, oxidizes iodide ion.

The oxidation of thiourea by the thyroid gland offers a method of studying the oxidizing mechanism of the thyroid gland.

I am indebted to Dr. Richard P. Keating, with whom these studies were initiated. Mrs. Frances Barkman provided able technical assistance.

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PHOSPHATASE OF BRAIN TISSUE*

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(Received for publication, May 19, 1950)

An enzyme of brain tissue, active in the removal of the terminal phosphate of adenosinetriphosphate, has been obtained in a purified condition. This enzyme was found to hydrolyze inorganic pyrophosphate but did not hydrolyze hexose diphosphate, phenyl phosphate, α -glycerophosphate, thiophosphate, or adenylic acid. The activity of the enzyme was dependent upon the presence of magnesium ions; in the absence of magnesium ions no activity could be detected. Glutathione (or cysteine) was essential for the demonstration of full activity. Enhanced activation of the enzyme was observed when the preparation was preincubated with magnesium ions together with glutathione. Preincubation with magnesium ions alone was relatively ineffective, even though glutathione was added to the test solution. Preincubation with glutathione alone allowed the preparation to attain almost the same activity as when preincubated with glutathione and magnesium ions. Nevertheless, no activity was observed unless magnesium ions were added.

Our interest in this enzyme was prompted by the belief that the Apyrase studied by Myerhof and associates (1) was a component of the nervous tissue or, more correctly, the transmission components of muscle. Our preparation has many of the properties of the preparation of Meyerhof and we have found that when our methods of isolation were applied to muscle a similar, if not identical, preparation was obtained. Further studies are under way and will be reported in the near future.

EXPERIMENTAL

Preparation of Enzyme—One pig brain (approximately 200 gm.) was homogenized with 500 ml. of cold distilled water in a Waring blender and the mixture was centrifuged. 1 volume of cold acetone was added to the supernatant solution and the mixture centrifuged. The precipitate was discarded and an additional volume of acetone was added. The precipitate was dissolved in 200 ml. of water and the solution dialyzed against frequent changes of distilled water for 24 to 48 hours. The precipitated material was removed by centrifugation and discarded. 4 vol-

* These studies were supported by grants from the United States Public Health Service.

umes of cold acetone were added to the supernatant solution and the mixture was allowed to stand at 5° overnight. The insoluble material was removed by centrifugation and discarded. Dilute acetic acid (0.1 N) was added dropwise and with stirring until precipitation was complete.

The precipitated material was redissolved in water by the addition of small amounts of solid sodium bicarbonate and the solution was dialyzed for 48 hours against distilled water. 1 volume of acetate buffer (pH 4.0) was added and the precipitate discarded. 2 volumes of ethanol were then added and the material that precipitated was discarded. 2 additional volumes of acetone were added and the precipitate was collected and dissolved in water. In most cases, the material precipitated as broken plates which were birefringent under polarized light.

TABLE I
Activation by Metal Ions

10 mg of sodium pyrophosphate were incubated for 10 minutes with an amount of enzyme represented by 0.03 mg. of N in a total volume of 10 ml. of 0.05 M histidine buffer, pH 7.1. The enzyme was preincubated 30 minutes with the metal ion and 10 mg. of glutathione. The concentration of the metal ion was 0.002 M.

Metal ion	Activity
	<i>mg. P</i>
None.....	0.00
Mg ⁺⁺	0.99
Co ⁺⁺	0.00
Zn ⁺⁺	0.01
Ca ⁺⁺	0.00
Fe ⁺⁺	0.00
Mn ⁺⁺	0.09

Under the conditions described by Meyerhof and associates (1), the material was found to have a Q_p with pyrophosphate as substrate of 90,000 to 100,000; with adenosinetriphosphate as substrate the Q_p was 150,000 to 200,000. Although these values are more than one order of magnitude greater than those reported by Meyerhof (1), there is reason to believe that the difference was due to the enhanced activation by preincubation with glutathione and magnesium ions rather than to a marked difference in the purity of the preparations. Preparations from muscle were found to have an activity similar to those from brain.

In the final solutions of 200 ml., the nitrogen content was approximately 0.03 mg. per ml. If the assumption was made that the nitrogen was protein nitrogen, approximately 40 mg. of enzyme were obtained from a pig brain.

Specificity of Metal Activation and Substrates—In Table I certain aspects

of the activation by metal ions are summarized. It is apparent that the effect of magnesium ions is quite specific; only manganous ions were observed to have a similar effect and, in this case, the effect was minimal.

The specificity of the enzyme was marked; only adenosinetriphosphate and pyrophosphate were found to be attacked. The hydrolysis of pyrophosphate was observed to proceed to completion, but with adenosinetriphosphate only one-half of the acid-labile phosphate was released during a period of prolonged incubation (90 minutes).

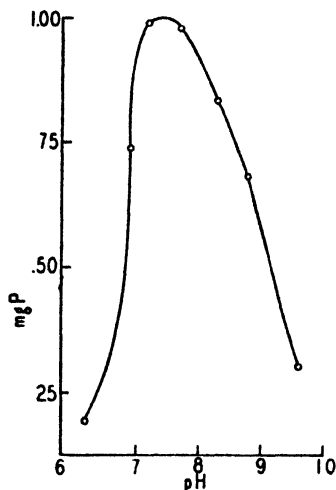


FIG. 1

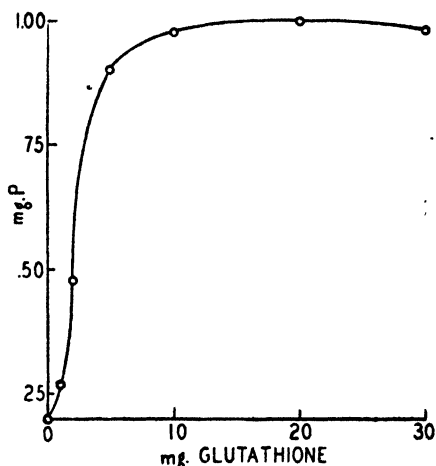


FIG. 2

FIG. 1. pH-activity relationship. 10 mg. of sodium pyrophosphate were incubated with an amount of enzyme represented by 0.03 mg. of N in a total volume of 10 ml. of 0.05 M histidine buffer with 0.005 M magnesium chloride and 10 mg. of glutathione. The enzyme was preincubated for 30 minutes before addition of the substrate. The time of incubation was 10 minutes.

FIG. 2. Effect of concentration of glutathione. The conditions were as in Fig. 1 except for the differences in concentration of glutathione. The pH was 7.1.

The effect of pH on the activity of the enzyme was studied; this study is summarized in Fig. 1.

Effects of Preincubation with Magnesium Ions and Glutathione—In Fig. 2 the effect of glutathione upon the activity of the enzyme is summarized. It is apparent that near optimal effects were obtained with a concentration of 1 mg. of glutathione per ml. of digest. In Fig. 3 the effects of various concentrations of magnesium ions and the effects of preincubation are summarized. Maximal or near maximal activity was obtained at concentrations of magnesium ions varying from 0.0025 to 0.0100 M; at the higher levels, some inhibition was observed. If the activity is plotted as a function of the concentration of magnesium ions, a mass law relationship is

evident (Fig. 4). When the curve was compared with similar theoretical curves for mass law relationships, it was found that the relationship was that of a combination of 4 (or perhaps 5) magnesium ions with 1 molecule of enzyme. Jenner and Kay (2) have ascribed the inhibition of kidney phosphatases by higher levels of magnesium to the formation of a substrate-magnesium-enzyme complex with 2 magnesium ions. From the

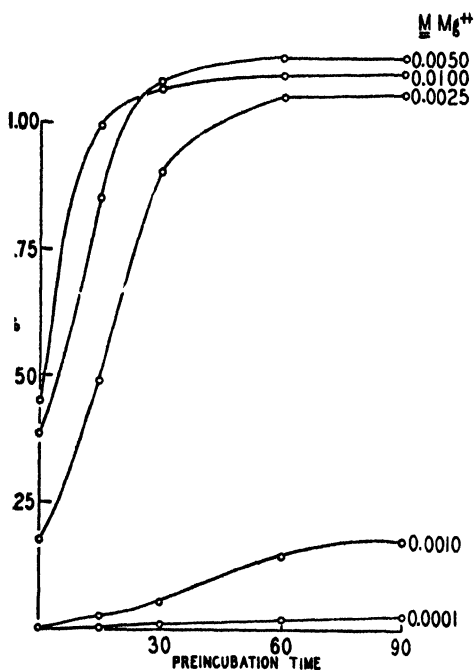


FIG. 3

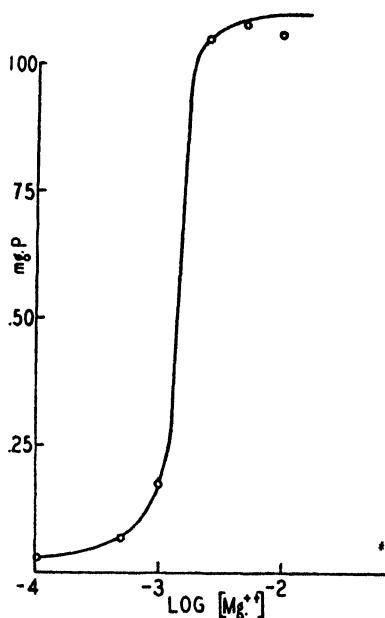


FIG. 4

FIG. 3. Relationship of concentration of magnesium ions to activity. The conditions were as in Fig. 2 except for the concentration of magnesium ions and time of preincubation.

FIG. 4. Effect of concentration of magnesium ions. The conditions were as in Fig. 3. Each sample was preincubated with magnesium ions and glutathione for 120 minutes before addition of the pyrophosphate.

data summarized in Fig. 5, it is seen that preincubation with glutathione alone was much more effective than preincubation with magnesium ions but was not as effective as preincubation with magnesium ions and glutathione. When the enzyme was incubated with magnesium ions alone for extended periods (8 to 10 hours), considerable activation was observed and, in most cases, the extent of activation could be anticipated by an extension of the lower curve in Fig. 5. However, the activation by magnesium

ions alone with prolonged incubation never approached more than 50 to 60 per cent of that obtained by the shorter periods of preincubation with magnesium ions and glutathione.

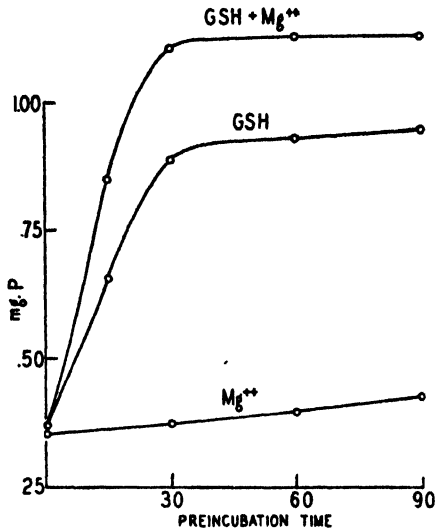


FIG. 5. Preincubation with and without magnesium ions. The conditions were as in the preceding experiments except that magnesium ions (0.005 M) and glutathione were added at the end of the preincubation periods in the lower curves.

DISCUSSION

It might be expected that the complete dependence of the activity of the phosphatase from brain upon the presence of magnesium ions would permit clear cut studies as to the function of the metal ion in the enzymatic process. Superficially, our results appear to be in agreement with the concept of the participation of the magnesium ion in the formation of a protein-metal-substrate complex, as has been suggested by Bauer (3) and by Jenner and Kay (2). However, the rôle of glutathione in the activation of the phosphatase by magnesium ions is not entirely clear. In agreement with the theory of metal coordination, it might be assumed that the formation of the protein-magnesium-pyrophosphate complex proceeds in two steps. The first step would be the reduction of disulfide groupings of the enzyme and the second step would be the combination of the magnesium ions with the protein, presumably at sulfhydryl groupings. There are, however, certain objections to this concept. First, greater activity is obtained when the enzyme is preincubated with magnesium ions and glutathione than when the enzyme is preincubated with glutathione alone. There is no reason to expect that the combination of

magnesium ions with the reduced protein would be a slow process. Second, when the enzyme was preincubated for extended periods with magnesium ions alone, the activity approached that obtained by preincubation with magnesium ions and glutathione. It is difficult to believe that magnesium ions can bring about the reduction of disulfide linkages of the protein. The slow rate of activation of the enzyme by magnesium ions alone would seem to suggest that profound changes in the configuration of the protein have taken place; the question as to whether or not the same profound changes are induced by glutathione cannot be answered from the available information.

It would appear that the basic assumptions, that the activation by glutathione is concerned solely with reduction of disulfide groupings of proteins and that the function of the metal is in the formation of a protein-metal-substrate complex, are much too simplified.

It is to be hoped that the availability of the phosphatase from brain tissue and of a simple substrate, pyrophosphate, will permit decisive studies as to the functions of the metal ion in the activation of the enzyme.

SUMMARY

A phosphatase of brain tissue has been obtained in a purified condition. The enzyme was found to hydrolyze inorganic pyrophosphate and to remove the terminal phosphate of adenosinetriphosphate. The activity of the enzyme was found to be dependent upon the presence of magnesium ions. Considerable activation of the enzyme was observed when glutathione was added together with the magnesium ions, but maximal activity was obtained only by preincubation of the enzyme with magnesium ions and glutathione.

The results were examined in terms of the concept of a protein-metal-substrate complex, as suggested by Bauer and by Jenner and Kay, and certain objections to the concept were noted.

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METABOLISM OF GLUTATHIONE

III. ENZYMATIC HYDROLYSIS OF CYSTEINYLGLYCINE*

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As a part of the study of the hydrolysis of glutathione, it became necessary to study the behavior of the enzyme responsible for the hydrolysis of cysteinylglycine (1). This enzyme has been obtained free from the enzyme responsible for the hydrolysis of the γ -glutamyl linkage of glutathione.¹ It was found that the enzyme was activated by manganous, ferrous, or cobaltous ions.

EXPERIMENTAL

Assay of Activity—Cysteinylglycine was prepared by the mild hydrolysis of glutathione with acid. The glutathione was hydrolyzed with 1.2 N hydrochloric acid for 60 minutes at 94°. Under these conditions, complete hydrolysis was indicated by the method of Sullivan and Hess (2) as adapted for the determination of cysteinylglycine and cystinyldiglycine (1). Attempts at isolation of the cysteinylglycine have failed. When cystinyldiglycine was formed (by oxidation with air in the presence of ferric ions), it could be isolated. When attempts were made to isolate the cysteinylglycine as the cuprous mercaptide, followed by decomposition of the cuprous mercaptide with hydrogen sulfide, the solutions were found to contain no cysteinylglycine, even though the nitroprusside test was strongly positive. It would appear that during the manipulations the material had been converted to the diketopiperazine. This conclusion was strengthened by the observations that, with prolonged hydrolysis or with standing, cysteinylglycine disappeared from solution without the loss of sulphydryl groupings (as determined by iodometric methods) or with the appearance of cysteine. Therefore, cystinyldiglycine was used as the standard in the determination of cysteinylglycine by the method of Sullivan and Hess. It should be mentioned that the cysteinylglycine as obtained from glutathione produced twice as much color as did cystinyldiglycine in this procedure.

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¹ Throughout this report CGase is used to designate the activity toward cysteinylglycine and GSHase to designate the activity toward glutathione.

The substrate was prepared immediately before use in the following manner: 250 mg. of glutathione were dissolved in 9 ml. of water in a test-tube, and 1.0 ml. of concentrated hydrochloric acid was added. The mixture was placed in a boiling water bath (94° at this altitude) and heated for 60 minutes. At the end of this period, the contents of the test-tube were transferred to a 25 ml. volumetric flask. The flask was cooled and the calculated amount of sodium hydroxide added. The final pH of the solution was adjusted to 7.1 to 7.5 and the contents of the flask were adjusted to volume.

Various amounts of the fresh solution were used to study the activity of the CGase. In most cases, 1 ml. of the substrate-solution, an aliquot of the preparation of enzyme, 1 ml. of 0.005 M manganous sulfate, 2 ml. of histidine buffer, 0.1 M, pH 7.3, and water to a total volume of 9.5 ml. were incubated at 37°. At the end of the incubation period, 0.5 ml. of 50 per cent trichloroacetic acid was added and the protein removed by filtration. Aliquots of the filtrate (1 ml. or less) were used in the determination. The color was estimated at 500, 540, and 580 μ . As has been explained (1), the per cent hydrolysis was indicated by the ratio of the optical density at 500 to that at 580 μ . The 500:580 ratio for cysteine (or cystine) was approximately 3.4; the ratio for cysteinylglycine (cystinylglycine) was approximately 1.3. There was a straight line relationship between the per cent of cysteine and the increasing ratio. Therefore, it was sufficient to calculate the 500:580 ratio in each case and, thus, from a graph, determine the per cent cysteine or per cent hydrolysis.

Preparation of Enzyme—In the kidney of the pig the CGase appears to have a physical as well as a functional relationship to the GSHase. It was found, however, that treatment with ethanol and chloroform would dissociate the two activities. As a result of the treatment with organic solvents, the CGase became dissociated from GSHase and required increasingly higher concentrations of ethanol for its precipitation from solution as the separation proceeded.

100 gm. of fresh pig kidney were homogenized with 100 ml. of 95 per cent ethanol at 0° and the mixture was centrifuged. The supernatant solution was discarded and the residue homogenized with 100 ml. of distilled water. When 1 volume of ethanol was added to the mixture, considerable CGase was precipitated. This CGase was apparently in combination with the GSHase. The precipitate was mixed with 200 ml. of water and was shaken vigorously in the cold with 65 ml. of chloroform. The mixture was centrifuged and the clear supernatant solution removed by suction. This solution was reasonably free from GSHase and contained a considerable amount of the original CGase activity. At this point, 5 volumes of ethanol were required to precipitate the active material. The precipitate

was dissolved in water and dialyzed against frequent changes of distilled water for 24 hours. The treatment with chloroform was repeated. The clear supernatant solution was lyophilized. Approximately 21 mg. of dried material were obtained. The procedure is summarized in Table I. The activity toward glutathione was determined in the presence of gluta-

TABLE I
Purification of CGase

A unit of CGase activity was described as that amount of enzyme necessary for the hydrolysis of 10 per cent of the substrate in 10 minutes. A unit of GSHase activity was defined as an amount of enzyme necessary to produce 1 mg. of cysteine (or cysteine, equivalent as cysteinylglycine) in 10 minutes from 10 mg. of glutathione and 5 mg. of glutamine.

Step	CGase	CGase	GSHase
	<i>total units</i>	<i>units per mg. N</i>	<i>total units</i>
Precipitation with alcohol	420,000	290	840
Treatment with chloroform	95,000	1400	145
Increase of alcohol from 4 to 5 volumes	46,000	1640	50
Treatment with chloroform	34,200	5350	4

TABLE II
Activation of CGase by Metal Ions

The metal ions were added as indicated and the mixture was preincubated for 1 hour before addition of the substrate. 0.001 mg. of protein N were present in each ml. of digest.

Metal ion	Concentration	Per cent hydrolysis
	<i>M</i>	
Mg ⁺⁺	0.0005	6.0
Ca ⁺⁺	0.0005	6.0
Pb ⁺⁺	0.0001	3.2
Co ⁺⁺	0.0001	18.8
Fe ⁺⁺	0.0005	23.0
Mn ⁺⁺	0.0005	34.5
Control.....		7.0

mine. Glutamine was found to activate the hydrolysis of glutathione by the combined system but has no effect on the activity of CGase (3).

pH-Activity and Metal Activation Relationships—In Table II the activation of the CGase by various metal ions is summarized. It is apparent that the activity is increased by the addition of manganous, cobaltous, or ferrous ions; other ions were either without effect or were inhibitory.

In Fig. 1 the effects of concentration of manganous ions on the activity of the enzyme are illustrated. It is apparent that the activation obeys a mass law relationship and that little or no activity is observed in the absence of manganous ions. It is interesting that the curve is that of a combination of 1 manganous ion with 2 molecules of enzyme; this behavior

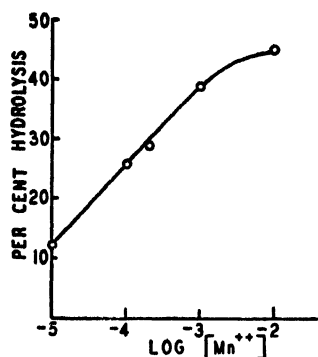


FIG. 1

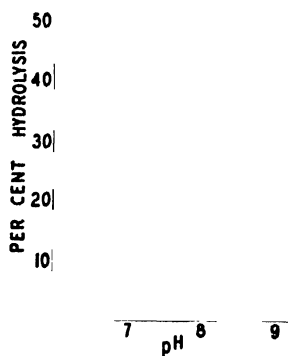


FIG. 2

FIG. 1. Relationship of concentration of manganous ion to activity. The experiments were made as described in the text with 0.001 mg. of protein N per ml. of digest. The enzyme was preincubated 2 hours with the manganous ions before addition of the substrate. The time of incubation was 10 minutes.

FIG. 2. Effect of pH on activity of CGase. 0.1 M histidine buffers were used as described in the text. 0.001 mg. of protein N was present in each ml. of digest.

TABLE III

Effect of Preincubation on Metal Activation

As indicated, 10 mg. of glutathione were present in the digests. 0.001 mg. of protein N was present in each ml. of solution and the incubation was at 37° for 10 minutes.

Time of preincubation	Per cent hydrolysis	Per cent hydrolysis with glutathione
<i>min.</i>		
0	22	5
30	37	9
60	46	12
120	52	15

is in contrast to that of the phosphatase of brain tissue, in which the curve was that of a combination of 4 (or 5) magnesium ions with 1 molecule of the enzyme (4). One interpretation of these results is that a dimerization of the enzyme has occurred; at this stage other explanations are possible and it would be premature to attempt to interpret the results solely on the basis of an association of protein molecules.

The effects of preincubation with manganous ions in the presence and in the absence of glutathione are illustrated in Table III. It is apparent that maximal activation was obtained in the absence of glutathione and that glutathione was inhibitory.

In Fig. 2 a study of the pH-activity relationship is illustrated.

DISCUSSION

The results we have obtained are suggestive of a physical, as well as of a functional, relationship between the GSHase and CGase of kidney tissue; the description of this relationship will be extended in subsequent reports. The activation of the CGase by manganous or cobaltous ions is probably not of a physiological nature; the activation by ferrous ions would appear to be the more logical mechanism if a metal ion is required for the activity of the enzyme *in situ*.

SUMMARY

The enzyme of pig kidney responsible for the hydrolysis of cysteinyl-glycine has been separated from the enzyme responsible for the hydrolysis of the γ -glutamyl linkage of glutathione. Manganous or ferrous ions were found to increase the activity of the enzyme.

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OROTIC ACID IN THE NUTRITION OF A STRAIN OF *LACTOBACILLUS BULGARICUS*

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In an investigation of the nutrition of various strains of *Lactobacillus bulgaricus*¹ an organism was obtained that would not grow on a complex medium containing casein hydrolysate, purines, pyrimidines, vitamins, a yeast extract, and clarified tomato juice. A wide diversity of natural products was tested for their ability to support the growth of *L. bulgaricus* O9, but only whey or related products² supported maximal growth of the organism.

Fractionation of these milk products led to the isolation of a biologically active crystalline compound which was identified as orotic acid.

When orotic acid was added to the basal medium and the tomato juice and yeast extract were omitted, no growth was obtained. Caswell *et al.* (1) reported that the tomato juice used in their medium for the estimation of vitamin B₁₂ with *Lactobacillus lactis* Dorner could be replaced by a mixture of fumaric acid and ethyl sodium oxalacetate. With *L. bulgaricus* O9 this mixture does not support optimal growth of the organism. If citric acid is added in addition to the fumaric acid and ethyl sodium oxalacetate, satisfactory growth is obtained.

EXPERIMENTAL

Organism—*L. bulgaricus* O9 was carried in a medium containing 50 mg. of yeast extract (Difco) and 1 ml. of filtered tomato juice per 10 ml. of skimmed milk. The organism was transferred once weekly into a medium containing 75 mg. of yeast extract (Difco), 1 ml. of the standard whey solution (see below), and 9 ml. of basal medium (Table I) per tube. Daily transplants were made. Inocula were prepared by centrifuging the cells from a daily transplant, washing with saline, and then resuspending in 10 ml. of 0.9 per cent saline. 1 drop of this suspension was used per tube as inoculum. Titratable acidity was measured after the tubes were incubated 40 hours at 37°.

¹ This culture was obtained from Dr. J. M. Sherman, Department of Bacteriology, Cornell University, Ithaca, New York.

² We are indebted to Mr. E. Malone of Sheffield Farms, Inc., for samples of No. 2 wash water and mother liquor concentrate.

Preparation of Standard Whey Solution—13 gm. of Borden's Starlac milk powder³ were suspended in 100 ml. of water, adjusted to pH 4.0 with hydrochloric acid, and then heated for 30 minutes on the steam bath. The solution was filtered and the filtrate diluted to 130 ml.

Initially such whey solutions were used in fractionation studies. Later a Sheffield Farms product, No. 2 wash water and mother liquor concen-

TABLE I
Composition of Double Strength Basal Medium

	gm.		γ
HCl-hydrolyzed casein	2.5	Riboflavin	250.0
Casitone, Difco	2.5	Thiamine	600.0
Glucose	5.0	Nicotinic acid	250.0
Lactose	5.0	<i>p</i> -Aminobenzoic acid	600.0
Sodium acetate	6.0	Folic acid	10.0
L-Cystine	0.100	Vitamin B ₁₂	0.3
DL-Tryptophan	0.200	Pyridoxine	250.0
Yeast extract, Difco	0.500	Pyridoxal	100.0
	mg.	Pyridoxamine	100.0
Asparagine	50.0	Pimelic acid	100.0
Adenine	5.00		ml.
Guanine	5.00	Tomato juice, clarified	50.0
Uracil	5.00	Salts A*	2.5
Xanthine	5.00	" B*	2.5
Thymine	5.00	Tween 80	1.0
	γ	<i>L. bulgaricus</i> concentrate	0.25†
Inositol	500.0	(Williams <i>et al.</i> (11))	
Choline	500.0	Distilled water to	250.0
Biotin	0.5	Adjust to pH 6.5	
Calcium <i>d</i> -pantothenate	250.0		

5 ml. of this medium were added per tube. The test solution was added and the volume adjusted to 10 ml.

* Snell, E. E., and Strong, F. M., *Ind. and Eng. Chem., Anal. Ed.*, **11**, 346 (1939).

† The amount that is necessary to produce maximal growth must be determined for each preparation.

trate, which was approximately twice as active as the whey solution prepared from the milk powder, was used as the source material.

Isolation of Orotic Acid—100 gm. of the No. 2 wash water and mother liquor concentrate were diluted to 1 liter with water and adsorbed with 25 gm. of Nuchar C115N⁴ for 1.5 hours at 25°. After filtering, the charcoal cake was washed with water and then eluted twice with 500 ml.

³ The Borden Company, 350 Madison Avenue, New York 17, New York.

⁴ West Virginia Pulp and Paper Company, 230 Park Avenue, New York 17, New York.

portions of 50 per cent ethanol containing 5 per cent (by volume) of concentrated ammonium hydroxide at 45–50° for 60 minutes. The eluates were combined and then stirred with 100 gm. of Super Filtrol⁵ for 0.5 hour. The Super Filtrol was removed by filtration and the filtrate concentrated to approximately 35 ml. The solution was kept at 4° and after standing several days the crystalline ammonium salt precipitated. This compound was converted to the acid by treatment with hot 2 N hydrochloric acid. After an additional crystallization from water the com-

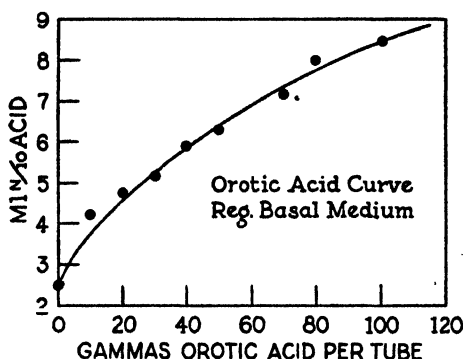


FIG. 1. Growth response to orotic acid on the basal medium containing yeast extract and tomato juice.

pound was dried at 135° in a vacuum. The product gave the following analyses:

$C_5H_4N_2O_4$. Calculated. C 38.48, H 2.56, N 17.94
 Found. " 37.97, " 3.37, " 17.68

A sample of orotic acid was prepared by the method of Nyc and Mitchell (2). The ultraviolet and infra-red absorption spectra of the natural and synthetic acids were identical.

Earlier isolation procedures included the removal of inert material at pH 5.0 with silver and the precipitation of the active material by silver at pH 8 to 9. The inclusion of this step does not materially affect the yield of orotic acid.

The response of the organism to orotic acid is illustrated graphically in Fig. 1.

Attempted Replacement of Orotic Acid by Known Compounds—A number of investigators have reported that uracil and orotic acid are interchangeable in certain biological systems (3, 4). Under the present conditions uracil at levels up to 1.0 mg. per tube elicits little if any growth response

⁵ Filtrol Corporation, 315 West Fifth Street, Los Angeles, California.

when used in lieu of orotic acid. Similarly, adenine, guanine, thymine, adenosine, guanosine, and a mixture of yeast nucleotides at concentrations of from 25 to 1000 γ per tube failed to cause any significant growth response.

It was of interest to determine whether or not orotic acid was incorporated as such into the cells of *L. bulgaricus* O9. For this experiment *L. bulgaricus* O9 was grown for 40 hours in the presence of 0.5 mg. of orotic acid per 10 ml. of medium. The cells were collected and autolyzed under toluene. Assay of these cells failed to show the presence of

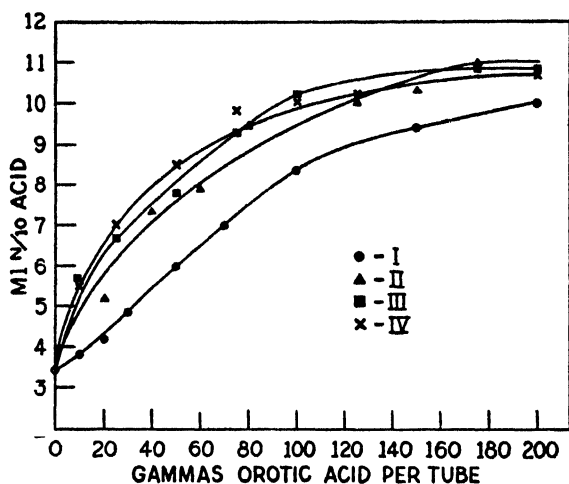


FIG. 2. Organic acids in the nutrition of *L. bulgaricus* O9. Curve I, sodium ethyl oxalacetate and fumaric acid. Curve II, sodium ethyl oxalacetate, fumaric acid, succinic acid, and citric acid. Curve III, sodium ethyl oxalacetate, fumaric acid, and citric acid. Curve IV, sodium ethyl oxalacetate, fumaric acid, citric acid, and pyruvic acid. All acids were added at a concentration of 4 mg. per tube with the exception of pyruvic acid which was added at 100 γ per tube.

orotic acid. The cells and culture fluid were then refluxed in 6 N hydrochloric acid, 2 N hydrochloric acid, or 2 N sodium hydroxide for 3 hours. The respective hydrolysates were assayed for orotic acid with negative results. Orotic acid when hydrolyzed under similar conditions was found to be stable. If the assumption is made that any orotic acid complex would be hydrolyzed by the above procedures, it is apparent that orotic acid *per se* has a transitory existence in the cell and is converted into an as yet unknown product.

Replacement of Tomato Juice and Yeast Extract with Known Compounds—Orotic acid was added to the basal medium containing no tomato juice or yeast extract, and various combinations of fumaric, citric, succinic,

maleic, malic, pyruvic, and glutaric acids, acetaldehyde, and ethyl sodium oxalacetate were tested for their ability to replace the activity of tomato juice and yeast extract.

Fumaric acid, citric acid, and ethyl sodium oxalacetate gave a response equivalent to that produced by the natural supplements. The addition of succinic or pyruvic acid in certain experiments gave a small additional growth increment (Fig. 2), but the results were not consistent. These variable effects may be associated with the differing physiological states of the inoculum.

DISCUSSION

It has previously been shown that orotic acid could substitute for uracil in satisfying the growth requirements of certain group C streptococci (3) and for two strains of *Neurospora* (4). One of the *Neurospora* mutants could also use cytosine or thymine. Uridine, cytidine, uridylic acid, and cytidylic acid were from 10 to 60 times as active as the free pyrimidines in supporting growth of the mutant strains. This is indicative of the conversion of the pyrimidines to the necessary nucleosides or nucleotides.

Chattaway (5) found that at times a liver fraction necessary for the growth of *Lactobacillus casei* could be replaced by orotic acid to give a partial response.

Our studies indicate that the principle present in milk products which is necessary for the growth of *L. bulgaricus* O9 is orotic acid and it could not under the conditions of the test be replaced by uracil, thymine, the purine nucleosides, or the nucleotides from yeast nucleic acid.

Studies with strains of *Neurospora* led Mitchell and Houlahan (6, 7) to the conclusion that neither uracil nor orotic acid is a normal precursor of uridine and that the carbon chain of the pyrimidines arises from oxalacetic acid (6). They suggested that possibly the ribose in the nucleosides is combined with a product derived from oxalacetic acid before ring closure takes place. Rat feeding experiments with isotopic uracil or thymine indicated that these pyrimidines were not incorporated into the nucleic acid (8). When isotopic orotic acid was fed to rats, an increase in the percentage of excess N^{15} was found in the pyrimidine nucleosides (9, 10), indicating the conversion of orotic acid but not uracil or thymine to components of the nucleic acids. The inability to find orotic acid in the cells of *L. bulgaricus* O9 grown in the presence of orotic acid indicates a conversion of this essential compound to some other product or products. If isotopic studies should indicate conversion to pyrimidine nucleosides or nucleotides, as in the rats, this organism might be a very useful tool for mechanism studies.

The necessity for oxalacetic, fumaric, and citric acids indicates inability of the organism to synthesize these compounds in sufficient quantity for its metabolic processes. If oxalacetic acid plays an important rôle in the synthesis of pyrimidine nucleosides, our data may indicate a general weakness of *L. bulgaricus* O9 in the early steps of synthesis of these compounds.

SUMMARY

A strain of *Lactobacillus bulgaricus* could be grown in a complex medium only if whey or related milk products were added. The active principle was isolated and identified as orotic acid.

No pyrimidines, purines, or related compounds tested could replace orotic acid.

The tomato juice and yeast extract could be replaced by a mixture of sodium ethyl oxalacetate, fumaric acid, and citric acid.

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GLUCONATE OXIDATION BY PSEUDOMONAS FLUORESCENS*

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The oxidation of glucose to gluconic acid and then to 2-ketogluconic acid by some species of *Pseudomonas* has been reported by Lockwood, Tabenkin, and Ward (1). Lockwood and Stodola (2) reported that one species, *Pseudomonas fluorescens* NRRL B-6, on further oxidation produced α -ketoglutaric acid as a major product. About 0.2 mole of α -ketoglutaric acid was produced per mole of glucose oxidized. Pyruvic and succinic acids were also found in small amounts.¹ We are currently interested in establishing this fermentation as an industrial source of α -ketoglutaric acid and have undertaken a detailed study of the chemical mechanism of its formation, with the object of providing information that will be helpful in directing the fermentation toward higher yields with a minimal production of by-products. The great ease with which α -ketoglutaric acid is metabolized has made study of the mechanism highly desirable.

The presence of gluconate and 2-ketogluconate during glucose oxidation by *P. fluorescens* B-6 suggests that the oxidation proceeds by way of the hypothetical hexose monophosphate shunt reaction sequence (3). The suggestion gains support by the finding of Lockwood and Nelson (4) that this organism will oxidize certain pentoses to corresponding pentonic acids and beyond. In the present work, the oxidation of gluconate by *P. fluorescens* was studied from the view-point of the hexose monophosphate shunt hypothesis. Conditions affecting the accumulation of pyruvate and α -ketoglutarate were investigated, and reaction balances of gluconate oxidation were obtained.

EXPERIMENTAL

Procedure for Conducting and Analyzing Fermentations—Carbon balances, and oxidation-reduction balances if possible, were desired at various stages of oxidation to insure that all major metabolic products would be accounted for. The use of converted suction flasks as fermentation vessels permits the determination of oxygen consumption and carbon dioxide evo-

* Part of the data in this paper was presented at the 116th meeting of the American Chemical Society at Atlantic City, September 21, 1949.

† One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

¹ Private communication.

lution, and also allows frequent aseptic sampling of gases and fermentation liquor during the prolonged incubation period necessary for optimal pyruvate and α -ketoglutarate accumulation. The fermentation vessel is shown in Fig. 1.

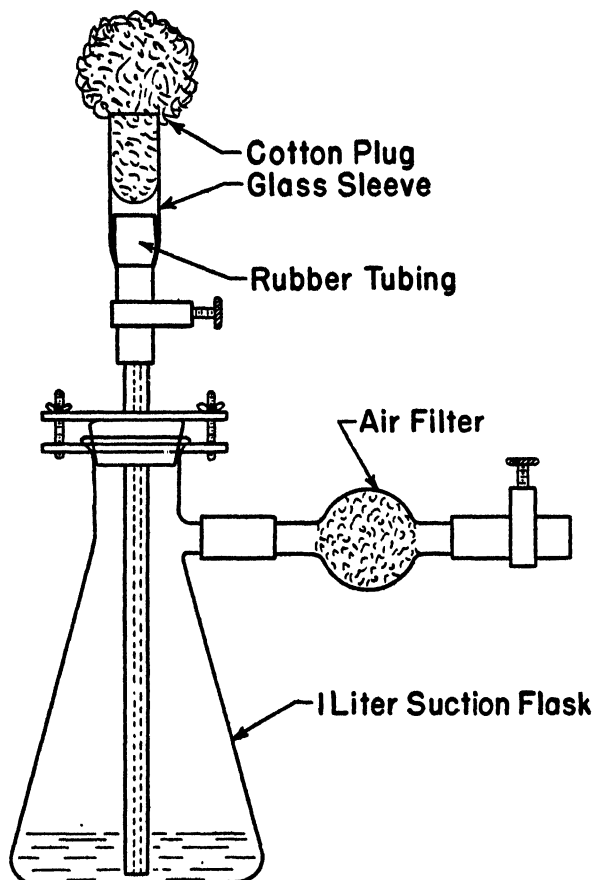


FIG. 1. Fermentation vessel

The medium (195 ml.) is prepared and sterilized in the flask. After inoculating with 5 ml. of 18 to 24 hour inoculum of *P. fluorescens* B-6 propagated in the same medium, the air in the flask is removed by aspiration through the air filter and replaced by oxygen. The oxygen content of the flask is calculated from the flask volume. The flask is placed on a reciprocating shaker and incubated at 27°. Periodically during incubation, a sample of the enclosed gas is removed and analyzed for carbon dioxide and oxygen according to ordinary methods of gas analysis. At

the same time, a sample (usually 10 ml.) of fermentation liquor is withdrawn aseptically. The remaining gas in the flask is replaced by oxygen, and incubation is continued.

The specimen of fermentation liquor is centrifuged. The cells are washed and analyzed for carbon according to Van Slyke and Folch (5). The supernatant liquor is analyzed for bound carbon dioxide by Van Slyke vacuum extraction. 2-Ketogluconate is determined as reducing power with the copper sulfate reagent of Somogyi (6). Pyruvate and α -ketoglutarate are measured simultaneously by a modification of the method of Friedemann and Haugen (7). The initial gluconate concentration is calculated from the weight of potassium gluconate used. Purity of the gluconate is verified by optical rotation and analysis for carbon and hydrogen.

Results

Oxidation of Purified Potassium Gluconate in Synthetic Medium—In order to reduce the amount of extraneous carbon compounds in the fermentations, it was desired to conduct gluconate oxidation in media of as simple composition as possible. Preliminary experiments indicated that oxidation could take place in liquid media containing potassium gluconate as the substrate and the sole source of carbon and ammonium sulfate as the sole source of nitrogen, monobasic potassium phosphate, and magnesium sulfate. No buffer is necessary, since the fermentations usually maintain themselves between pH 6.5 and 7.2. Gluconate oxidation characteristically begins with rapid formation of 2-ketogluconate in essentially stoichiometric yield. As 2-ketogluconate itself is oxidized, carbon dioxide, α -ketoglutarate, and pyruvate appear. However, the rate of fermentation as well as quantities and proportions of products in early experiments was dependent on the specimen of potassium gluconate employed, even though the specimens were essentially pure from an analytical standpoint. It appeared that trace impurities in the gluconate specimens were affecting the course of fermentation.

To investigate this possibility, a quantity of potassium gluconate was purified as thoroughly as possible by repeated recrystallization by using infusorial earth and activated charcoal. When oxidized in a medium containing 50 μ M of gluconate, 15 μ M of nitrogen (1 gm. atom per mole), 4 μ M of phosphate, and 1 μ M of magnesium sulfate per ml., this purified gluconate was oxidized rapidly to 2-ketogluconate. Further oxidation was very slow, requiring about 12 days for complete utilization of 2-ketogluconate. Pyruvate accumulated in a yield of 0.7 mole per mole of 2-ketogluconate oxidized. No α -ketoglutarate appeared. About 90 per cent of the initial carbon was accounted for as pyruvate, carbon dioxide, and cells.

Effect of Adding Iron—Molecular oxygen acts as an ultimate hydrogen acceptor in these gluconate oxidations. It was felt that the slow rate of oxidation of purified gluconate beyond 2-ketogluconate might be due to a lack of iron for the synthesis of iron-containing respiration catalysts. The addition of 0.02 μM of iron as ferrous ammonium sulfate per ml. to the above medium resulted in very marked stimulation of gluconate oxidation. The initial 50 μM of gluconate were now oxidized so rapidly that no 2-ketogluconate remained at 24 hours. No pyruvate was produced. Carbon dioxide and cells were the only major products.

Additional potassium gluconate (to give 100 μM per ml.) was supplied to the organisms at 48 hours and was oxidized rapidly to 2-ketogluconate. At first, the latter was oxidized only to carbon dioxide, but at 120 hours α -ketoglutarate and at 160 hours pyruvate began to accumulate. Both substances disappeared when the 2-ketogluconate was exhausted.

The extent of stimulation by iron was unchanged between 0.002 and 0.02 μM of iron per ml., which is the range tested. This range is approximately equivalent to between 0.1 and 1 part per million. The marked stimulation brought about by trace quantities of iron demonstrates the marked dependence of over-all metabolism on an adequate hydrogen transport system. It has been noted that in the absence of iron the cells, when centrifuged from the medium, have a very pale yellow color and that the medium contains sufficient pigment of a fluorescent nature to give a marked greenish yellow color. The pigment is almost absent in fermentations containing iron, while the cell crop has a pink flesh-like color. This suggests that in the absence of iron the organisms are able to synthesize an alternative hydrogen transport system having limited capacity.

Two-Stage Gluconate Oxidations—In subsequent experiments under these fermentation conditions, it was found that net cell production, as measured by analyses for cellular carbon, was confined to the very early part of the oxidation processes, the phase in which gluconate was being oxidized completely to carbon dioxide. Accumulation of α -ketoglutarate and pyruvate did not begin until net cell production had ceased. This finding makes it possible to separate the phase of cell proliferation from the phase during which pyruvate and α -ketoglutarate are accumulating, so that carbon and oxidation-reduction balances can be obtained in which cell production is eliminated. This is done by conducting the fermentations in two stages.

Cell proliferation is restricted to Stage I by balancing the amounts of potassium gluconate and nitrogen in the medium so that, except for cell production, carbon dioxide will be the only major product and no significant quantities of metabolic intermediates will accumulate. A gluconate level of 40 μM per ml. is preferred for this stage since the amount

of oxygen supplied in one filling of the flask is adequate, with slight excess, for complete oxidation. Although this small amount of gluconate is usually oxidized to carbon dioxide within 24 hours, Stage I is continued to 48 hours to provide excess time.

Stage II is begun at 50 hours by adding fresh potassium gluconate. This is oxidized rapidly (usually within 16 to 24 hours) to 2-ketogluconate. If the proper level of nitrogen has been supplied in Stage I, α -ketoglutarate accumulation now begins immediately, and pyruvate accumulation begins thereafter. If 2-ketogluconate disappears from the medium during active Stage II metabolism, the amounts of accumulated pyruvate and α -ketoglutarate decrease and may disappear also. The cellular carbon level remains unchanged, decreasing slightly in late phases of Stage II, apparently because of autolytic changes. Thus reaction balances of Stage II oxidation need not include cell proliferation.

Effect of Nitrogen Level—In two-stage experiments, the amounts of α -ketoglutarate and pyruvate accumulating, and to some degree the time at which the accumulations begin, are dependent on the amount of nitrogen furnished for cell proliferation. The optimal nitrogen level is about $7.5 \mu\text{M}$ per ml. At low nitrogen levels (about $4 \mu\text{M}$ per ml.) too few cells are produced to promote Stage II oxidation beyond 2-ketogluconate before fermentation ceases. At high levels (about $15 \mu\text{M}$ per ml.), cell proliferation continues well into Stage II, and the accumulation of pyruvate and α -ketoglutarate is delayed and decreased.

Fermentation Balances in Gluconate Oxidation during Pyruvate and α -Ketoglutarate Accumulation—A typical two-stage oxidation experiment in which the nitrogen and Stage I gluconate levels were chosen to bring about accumulation of high pyruvate and α -ketoglutarate levels, in relation to the 2-ketogluconate consumed, will now be described in greater detail. For Stage I, the medium contained $40 \mu\text{M}$ of potassium gluconate, $7.5 \mu\text{M}$ of nitrogen, $0.005 \mu\text{M}$ of iron as ferrous ammonium sulfate, $4 \mu\text{M}$ of monobasic potassium phosphate, and $1 \mu\text{M}$ of magnesium sulfate per ml. For Stage II, sufficient potassium gluconate was added at 50 hours to give a level of $100 \mu\text{M}$ per ml. The oxidations proceeded as shown in Fig. 2. α -Ketoglutarate accumulation began within 24 hours and pyruvate accumulation at about 48 hours after Stage II was begun. At 216 hours after inoculation, the pyruvate level decreased and was accompanied by an increase in the α -ketoglutarate level. This phenomenon has been observed in about half of the oxidations studied; in other cases, the levels of both substances remain approximately constant after reaching their highest point, providing that some 2-ketogluconate is still present.

Balance data for this fermentation are given in Table I. No 2-ketogluconate, pyruvate, or α -ketoglutarate was present at the end of Stage

I. 27 μM of carbon per ml. remained unaccounted for. This carbon represents soluble carbon such as peptides, products of cell autolysis, pigments, and other unidentified substances. At 216 hours, 23 μM of α -ketoglutarate and 54 μM of pyruvate had accumulated during utilization of 83 μM of 2-ketogluconate, and 18 μM of Stage II carbon remained unaccounted for. At 288 hours, 36 μM of α -ketoglutarate and 42 μM of pyruvate were present from the utilization of 90 μM of 2-ketogluconate, and 12 μM of Stage II carbon were unaccounted for. The relation of these

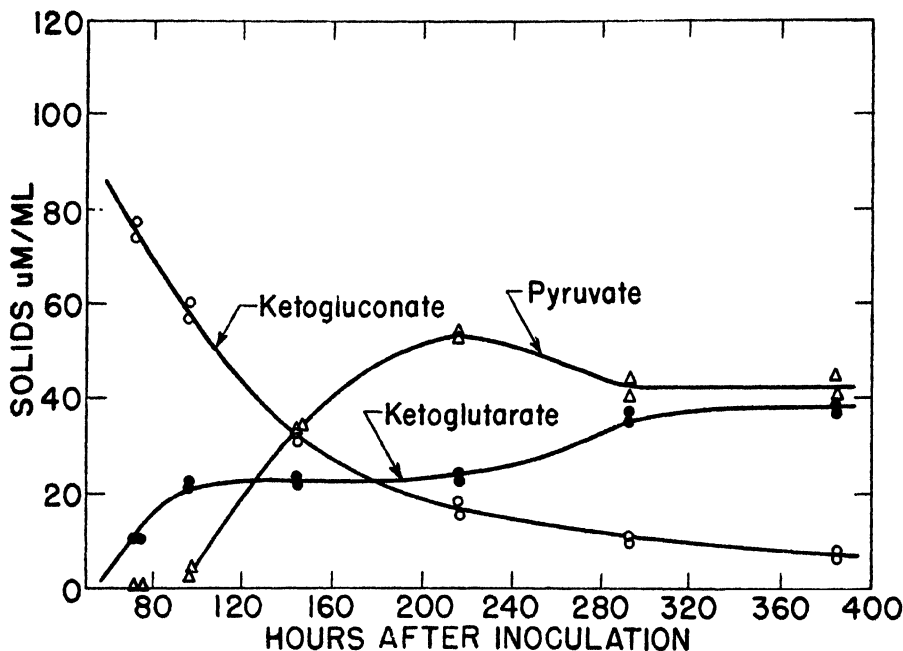


FIG. 2. Progress of gluconate oxidation in Stage II

results to contemporary hypotheses of carbohydrate oxidation will be discussed presently.

Search for Reducing Carbohydrates Other Than 2-Ketogluconate—According to the hexose monophosphate shunt oxidation hypothesis, a series of reducing carbohydrates, in phosphorylated form, should occur as intermediates in glucose or gluconate oxidation. If it is assumed, in keeping with this hypothesis, that the extracellular appearance of stoichiometric quantities of 2-ketogluconate in these fermentations results from its excretion by the cell after dephosphorylation of 6-phospho-2-ketogluconate, then it would appear probable that other reducing substances of the shunt sequence may be excreted in the same manner. These substances might be pentose, 2-ketopentionate, tetrose, 2-ketotetronate, and triose.

Several series of experiments were conducted in which reducing substances other than 2-ketogluconate were sought. Two-stage fermentations of the above type, and single stage fermentations of 150 μM of gluconate per ml. at a nitrogen level of 15 μM per ml., with and without iron, were analyzed at the peak of reducing power and at intervals thereafter. In all cases, the optical rotation of the fermentation liquors coincided with that which would be obtained if 2-ketogluconate were the only reducing substance present. The liquors were passed through cation and anion exchange columns. Less than 0.1 per cent of the reducing power appeared in the effluent, indicating that no neutral reducing compounds were present.

TABLE I
Fermentation Balances in Gluconate Oxidation in Presence of Iron and Limited Nitrogen

		After inoculation		
		Stage I	Stage II	
		48 hrs.	216 hrs.	288 hrs.
Substrates	Gluconate, μM per ml.	40	100	100
	Oxygen, μM per ml.	162	246	264
Products	Cell carbon, μM per ml.	40	0	0
	2-Ketogluconate, μM per ml.	0	17	10
	α -Ketoglutarate, " " "	0	23	36
	Pyruvate, μM per ml.	0	54	42
	Carbon dioxide, μM per ml.	173	203	222
	" unaccounted for, μM per ml.*	27	18	12
	" accounted for, per cent*	89	97	98
Oxidation-reduction balance, $\times 100^*$			90	90

* These values refer only to substances of the corresponding stage. Carbon unaccounted for in Stage I is disregarded in Stage II values.

It was concluded that the reducing substance detected during gluconate oxidation consists solely of 2-ketogluconate.

DISCUSSION

From these experiments, it appears that the accumulation of α -ketoglutarate and pyruvate may represent an abnormal or impaired mode of metabolism in gluconate oxidation by *P. fluorescens* B-6. Normal oxidation apparently constitutes complete oxidation of gluconate to carbon dioxide. Under our experimental conditions, complete oxidation was found early in the fermentation, and especially during the period when cell proliferation was most active. In time, the complete pathway appears to suffer an impairment or series of impairments, so that incompletely oxidized carbon residues, α -ketoglutarate and later pyruvate, accumulate

successively. It thus seems probable that these substances are intermediates in the normal pathway, whose further oxidation has been throttled or blocked entirely, but it is nevertheless possible that their accumulation represents a diversion from the normal metabolic mechanism.

Superficial similarity between glucose oxidation by *P. fluorescens* B-6 and the hypothetical hexose monophosphate shunt reaction sequence, reviewed by Potter (3), has been mentioned earlier. This similarity is based on the transient successive appearance of gluconate and 2-ketogluconate during glucose oxidation by the bacteria, and the ability of these organisms to oxidize pentoses to pentonic acids. Evidence for further conformity to the hypothesis was sought by searching for copper-reducing carbohydrates other than 2-ketogluconate during fermentation. None could be detected. This does not, of course, exclude the possibility that such reducing compounds are formed during gluconate oxidation, since metabolic intermediates are, in general, retained within the cell. Thus, direct evidence on the immediate fate of 2-ketogluconate was not obtained.

Information on the fate of 2-ketogluconate in relation to the contemporary shunt hypothesis may be deduced indirectly, however, from the yields of pyruvate and α -ketoglutarate obtained. According to the contemporary hypothesis, pyruvate would arise from the postulated 3-carbon residue and its production would be accompanied by evolution of 3 molecules of carbon dioxide per molecule of 2-ketogluconate oxidized. α -Ketoglutarate would be considered to arise during further oxidation of pyruvate. The latter assumption is supported by the apparent transformation of pyruvate into α -ketoglutarate during late phases of our experiments.

However, if only the carbon atoms of pyruvate are involved in α -ketoglutarate formation, then pyruvate plus α -ketoglutarate should account for less than 3 of the carbon atoms of dissimilated 2-ketogluconate. Instead, from data given in Table I at 216 and 288 hours, it is calculated that 3.34 and 3.40 carbon atoms, respectively, of dissimilated 2-ketogluconate are accounted for. We consistently obtain values between 3.2 and 3.6 in calculations of this type. It should be noted that both pyruvate and α -ketoglutarate are transient substances and that their accumulation is dependent on the balance between formation and destruction. It is possible that α -ketoglutarate destruction was not blocked completely in our experiments and that greater amounts than those which accumulated may have been formed.

Several explanations for these findings are possible. If 2-ketogluconate is dissimilated to form pyruvate and 3 molecules of carbon dioxide as postulated in the contemporary shunt hypothesis, then some of the carbon

dioxide produced must be taken up in further oxidation of pyruvate. Alternatively, instead of proceeding according to the contemporary hypothesis, 2-ketogluconate dissimilation may yield a 3-carbon or a 2-carbon fragment in addition to pyruvate, and α -ketoglutarate may arise by condensation of pyruvate with these fragments.

Additional experiments now under way should lead to further information on this fermentation mechanism.

SUMMARY

1. Conditions for the oxidation of gluconate by *Pseudomonas fluorescens* in synthetic medium, leading to the accumulation of high levels of pyruvate and α -ketoglutarate in relation to the gluconate oxidized, are described. Trace quantities of iron cause marked stimulation of gluconate oxidation. The accumulation of α -ketoglutarate and pyruvate is dependent on the level of nitrogen provided for cell proliferation.

2. The yields of α -ketoglutarate and pyruvate obtained are discussed in relation to contemporary hypotheses of gluconate oxidation suggested by the presence of 2-ketogluconate, α -ketoglutarate, and pyruvate among the oxidation products.

The author is indebted to Mr. Eugene S. Sharpe for assistance in performing the experiments.

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PHOSPHOMANNOSE ISOMERASE*

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Jephcott and Robison (1) isolated mannose-6-phosphate as a product of fermentation of mannose by dried yeast. They observed a greater accumulation of this ester at 38° than at 20° and suggested that the isomerization of mannose-6-phosphate was inhibited at the higher temperature. The occurrence of isomerization in the fermentation of mannose was indicated by the observation of Young (2) that the three fermentable hexoses formed the same hexose diphosphate when incubated with yeast juice and with inorganic phosphate.

An isomerase which catalyzes the equilibrium between glucose-6- and fructose-6-phosphate was first demonstrated by Lohmann (3) with extracts prepared from yeast, muscle, and other mammalian tissues. Somers and Cosby (4) found a similar enzyme in pea meal. It seems clear that this enzyme is widely distributed in nature.

The existence of a separate phosphomannose isomerase has been reported (5). The present paper is concerned with the partial purification of this enzyme from extracts of rabbit muscle and the equilibrium catalyzed by it.

Materials and Methods

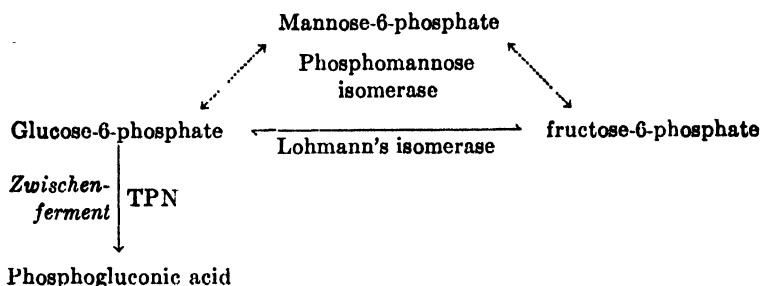
Mannose-6-phosphate was prepared by the action of adenosinetriphosphate (ATP) on mannose (Pfanstiehl's c.p. special quality) in the presence of crystalline yeast hexokinase (6). Glucose-6-phosphate, prepared from potato starch (7), was kindly supplied by Dr. T. Posternak. Fructose-6-phosphate was prepared from calcium fructose-1,6-diphosphate (Schwarz Laboratories) by a modification of the method of Neuberg *et al.* (8). The monophosphate was kindly furnished by Dr. J. F. Taylor. Triphosphopyridine nucleotide (TPN) was prepared, in collaboration with Dr. D. H. Brown, from horse liver by a modification of the method described for the preparation of TPN from red cells (9).

* This work was supported by a grant from the Nutrition Foundation, Inc. From the thesis presented by M. W. Slein to the Board of Graduate Studies, Washington University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Lohmann's isomerase was partially purified from rabbit muscle extract according to an unpublished method of Dr. A. A. Green. Glucose-6-phosphate dehydrogenase (*Zwischenferment*) was prepared from brewers' yeast (Anheuser-Busch) by a slight modification of the method of Negelein and Gerischer (10). Contaminating hexokinase was removed by repeated isoelectric precipitation of the dehydrogenase at pH 4.8 and by allowing solutions to stand for several days at 0°.

The isomerization of mannose-6-phosphate or of fructose-6-phosphate was followed spectrophotometrically by combining the appropriate isomerase systems with *Zwischenferment*, as indicated in the accompanying diagram.



Only glucose-6-phosphate is oxidized by TPN in the presence of *Zwischenferment*, and any reaction which results in the formation of glucose-6-phosphate may be detected in a spectrophotometer at 340 mμ.¹ The rate of such a reaction may be followed directly, provided that other enzymes are present in amounts sufficiently large so that the enzyme, the activity of which is to be determined, limits the over-all rate of the system. Since the oxidation of glucose-6-phosphate by TPN is irreversible, it may be used for the quantitative estimation of glucose-6-phosphate or its precursors by allowing the reaction to proceed to completion.

Trichloroacetate strongly inhibits *Zwischenferment*. For this reason, 2.5 per cent HgCl₂ in 0.5 M HCl was used for deproteinization, followed by treatment with H₂S, aeration, and neutralization, whenever protein-free filtrates were to be added to the *Zwischenferment* system for analysis.

Better than 95 per cent agreement was obtained between the enzymatic estimations of hexose-6-phosphates in the spectrophotometer and estimations based on phosphate analyses, as shown in Fig. 1. The horizontal lines represent optical densities expected by calculation from phosphate analyses and the curves show the rates at which TPN reduction approaches these values.

¹ The value of 6.22×10^4 sq. cm. per mole (for a cell of 1 cm. length) has been used as the molecular extinction coefficient for reduced TPN (11).

The spectrophotometric method of hexose phosphate estimation was further checked by a determination of the equilibrium established between glucose-6-phosphate and fructose-6-phosphate in the presence of purified Lohmann's isomerase. An aliquot of the neutralized mercury-free filtrate was analyzed for total hexose monophosphate by the spectrophotometric method. Fructose-6-phosphate was estimated colorimetrically (12) after it had been established that this ester gives 79 per cent of the color given by free fructose when heated with the resorcinol reagent for 10 minutes at 80°. At equilibrium at pH 7.4 and 30°, 30 per cent of the total hexose monophosphate was present as fructose-6-phosphate,

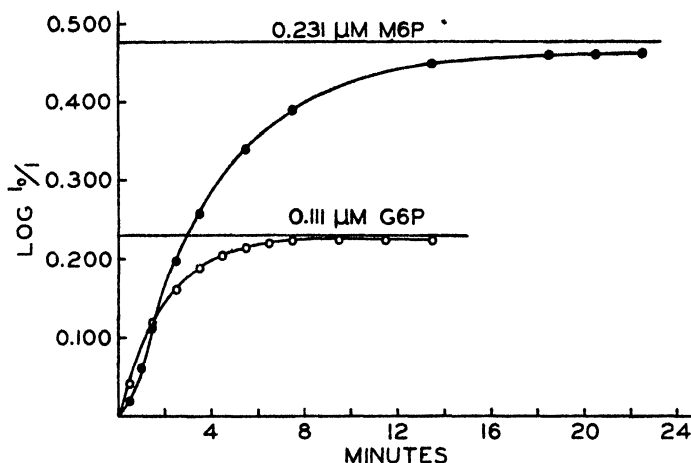


FIG. 1. Spectrophotometric determination of glucose-6-phosphate (G6P) and mannose-6-phosphate (M6P) with TPN, *Zwischenferment*, and, in the case of M6P, phosphomannose isomerase. The molar concentrations were calculated from P analyses and were converted to optical densities, as represented by the horizontal lines.

giving a ratio, glucose phosphate to fructose phosphate, of 2.33. This agrees with the values reported by Lohmann (3).

The following test system was used for estimation of phosphomannose isomerase activity in the Beckman spectrophotometer at 340 $m\mu$: 1.0 ml. of 0.05 M veronal, pH 8, 0.1 ml. of TPN (0.3 μM), 0.2 ml. of *Zwischenferment*, 0.2 ml. of 0.1 M $MgCl_2$, 0.1 ml. of phosphomannose isomerase, and 1.35 ml. of H_2O . After the initial optical density was determined, 0.05 ml. of mannose-6-phosphate (1 μM) was added and the rate of TPN reduction was followed for several minutes. 1 unit of activity was defined as a unit change in $\log I_0/I \times 10^3$ per minute when maximum velocity had been attained. A slight lag period occurred during most tests, but

the method proved to be sufficiently accurate for purposes of fractionation. Proportionality between enzyme concentration and phosphomannose isomerase activity is shown in Fig. 2.

Inorganic orthophosphate was determined according to Fiske and Subbarow (13) and protein according to Weichselbaum (14).

EXPERIMENTAL

Preparation and Properties of Phosphomannose Isomerase—Rabbit muscle was passed through a meat grinder in the cold, extracted twice with 1 volume of cold 0.02 M KOH, and strained through gauze. Partial purification of the enzyme was achieved by fractionation of the crude extract with a solution of $(\text{NH}_4)_2\text{SO}_4$ which had been saturated at 25° and adjusted to about pH 7.8 with concentrated NH_4OH . It was found that a fraction

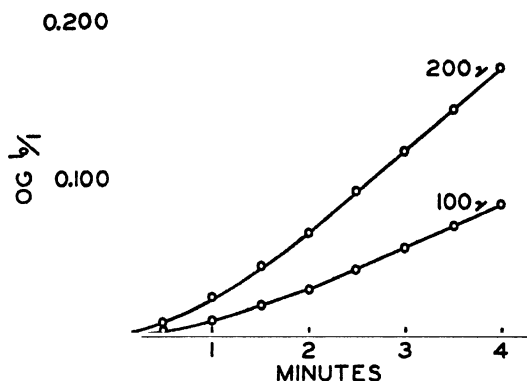


FIG. 2. Spectrophotometric determination of phosphomannose isomerase activity. Purified enzyme corresponding to 100 and 200 γ of protein was present per 3 ml.

obtained between 0.45 and 0.55 saturation with $(\text{NH}_4)_2\text{SO}_4$ usually had the highest activity.

Table I shows the result of subfractionating the protein obtained between 0.45 and 0.55 saturation with $(\text{NH}_4)_2\text{SO}_4$. Phosphomannose isomerase activity tends to be concentrated in the fractions precipitated below half saturation with $(\text{NH}_4)_2\text{SO}_4$. Two subfractions were combined and refractionated and gave material approximately 6 times as active as that of the original fraction.

Further purification was obtained by removing protein which precipitated during dialysis for several hours against cold distilled water. The best fraction so far obtained had an activity of about 1000 units per mg. of protein, which corresponds to a 12-fold purification over that of the original $(\text{NH}_4)_2\text{SO}_4$ fraction.

The enzyme is rather stable and withstands prolonged dialysis against cold 0.02 M NaHCO_3 . A solution of the enzyme may be stored in the frozen state for several weeks without appreciable loss of activity.

Separate Nature of Two Isomerases—By means of the enzymatic, spectrophotometric test system it is possible to show that purified Lohmann's isomerase from rabbit muscle does not catalyze the isomerization of mannose-6-phosphate. The opposite, however, has not been accomplished, namely to prepare phosphomannose isomerase of muscle free of Lohmann's isomerase. Purified preparations of *Zwischenferment* from brewers' yeast were found to contain varying amounts of an isomerase which acted on fructose-6-phosphate but not on mannose-6-phosphate.² The experiment in Fig. 3 illustrates these findings.

In Fig. 3, Curve I, the substrate was fructose-6-phosphate. The initial slow rate of TPN reduction was due to the presence of a small amount of

TABLE I
Purification of Phosphomannose Isomerase

Subfractionation of original 0.45 to 0.55 saturated ammonium sulfate fraction of rabbit muscle extract.

	Original	1	2	3	4	1 + 2	A	B	C	D
	0.45-0.55	0-0.47	0.47-0.50	0.50-0.53	0.53-0.56		0-0.45	0.43-0.47	0.47-0.50	0.50-0.60
Units per mg. protein.....	86	176	188	32	7	185	534	470	160	0
% units.....		17	53	9	<1		38	40	29	0
% protein.....		8	24	23	8		13	16	34	23

Lohmann's isomerase in the *Zwischenferment* preparation. Purified Lohmann's isomerase from rabbit muscle was added at the 3rd minute, after which there was a rapid reduction of TPN corresponding to the conversion of fructose-6-phosphate to glucose-6-phosphate.

Curve II is initially the same as Curve I. At 3.5 minutes, purified phosphomannose isomerase (0.1 mg. of protein) was added. This fraction contained about half as much of Lohmann's isomerase as was added in the experiment represented by Curve I.

Curve III illustrates an experiment in which the substrate was mannose-6-phosphate. During the first 4 minutes, *Zwischenferment* (contain-

² The maceration juice for this preparation was obtained by allowing dried yeast to autolyze for 6 hours at 37°. There is some indication that the phosphomannose isomerase is temperature-sensitive, and this may be the reason why it could not be detected in several other fractions obtained during the preparation of *Zwischenferment*.

ing isomerase as an impurity) and purified Lohmann's isomerase were present. The very low activity shows that Lohmann's isomerase from muscle and from yeast has practically no ability to catalyze the isomerization of mannose-6-phosphate. The addition of phosphomannose isomerase (0.1 mg. of protein) at 4 minutes resulted in a rapid isomerization of mannose-6-phosphate.

Whether the primary product of mannose-6-phosphate isomerization is glucose-6-phosphate or fructose-6-phosphate has not been established because all fractions of phosphomannose isomerase so far tested contained

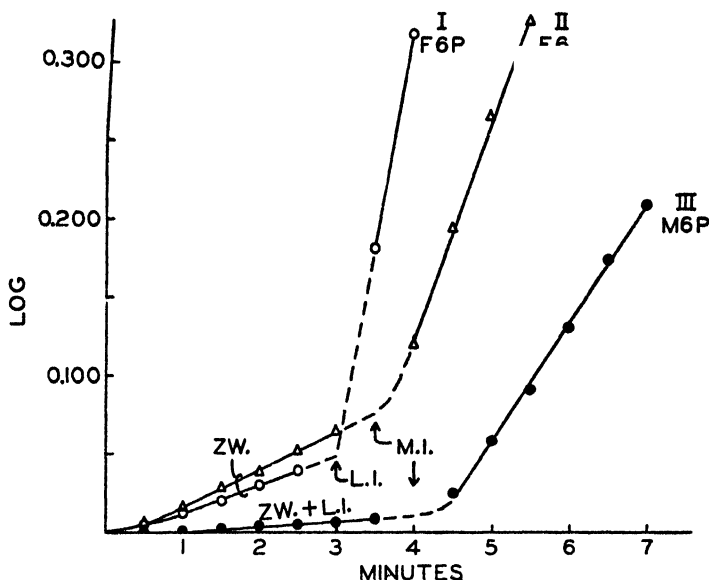


FIG. 3. Demonstration of the separate nature of Lohmann's isomerase (*L. I.*) and phosphomannose isomerase (*M. I.*). The substrate was fructose-6-phosphate (F6P) in Curves I and II and mannose-6-phosphate (M6P) in Curve III. *Zwischenferment* and TPN were present initially in all three cases, and Lohmann's isomerase in Curve III. Time of addition of isomerases indicated by arrows.

enough of Lohmann's isomerase to establish a rapid equilibrium between glucose-6- and fructose-6-phosphate. An illustrative experiment is shown in Fig. 4. Fructose-6-phosphate was determined colorimetrically in aliquots of the reaction mixture during incubation of phosphomannose isomerase with either glucose-6- or mannose-6-phosphate as substrate. The isomerization of mannose-6-phosphate proceeded smoothly and resulted in an equilibrium mixture containing close to 25 per cent fructose-6-phosphate. In the case of glucose-6-phosphate there was an initial overshooting of the equilibrium because of the high activity of Lohmann's isomerase

in this preparation. During the first minute 29 per cent fructose-6-phosphate was formed, followed by a slow decrease to 25 per cent, the latter phase corresponding to the activity of phosphomannose isomerase.

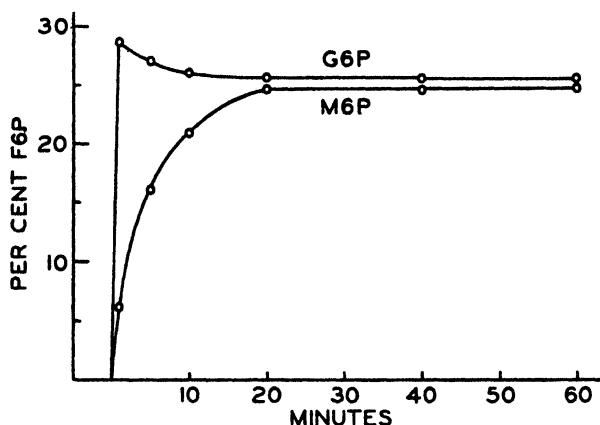


FIG. 4. Rate of conversion of glucose-6-phosphate (G6P) or mannose-6-phosphate (M6P) to fructose-6-phosphate (F6P) at pH 7.4, 30°, by a purified preparation of phosphomannose isomerase. Fructose-6-phosphate is expressed as per cent of the total hexose monophosphate concentration.

TABLE II

Equilibrium Established in Presence of Lohmann's Isomerase and Phosphomannose Isomerase at pH 7.4, 30°

The abbreviations, G6P, M6P, and F6P, refer to glucose-, mannose-, and fructose-6-phosphate, respectively.

Ester added	Per cent at equilibrium			Ratio, G6P:F6P
	G6P	F6P	M6P	
G6P..	56.4	26.0	17.6	2.17
M6P.....	55.7	25.7	18.6	2.17
F6P.....	60.0	24.7	15.3	2.43
Average	57.4	25.5	17.1	2.25

Ratio expected from Lohmann's isomerase 2.33

Equilibrium—Fig. 4 shows the attainment of equilibrium in the presence of both isomerases when either glucose-6-phosphate or mannose-6-phosphate is added as substrate. The relative proportion of the three monophosphates in the equilibrium mixture was determined as follows. The protein-free filtrates were analyzed for total hexose monophosphate

with *Zwischenferment*, TPN, and both isomerases. Glucose-6-phosphate plus fructose-6-phosphate was estimated in the presence of only Lohmann's isomerase. The amount of mannose-6-phosphate was calculated from the difference between these two values. Fructose-6-phosphate was determined in another aliquot by the colorimetric resorcinol method. This allowed the amount of glucose-6-phosphate to be calculated by difference.

Table II shows that (within experimental errors) the same equilibrium was reached, no matter which of the three hexose phosphates was added initially. The average ratio of glucose phosphate to fructose phosphate (2.25) is in agreement with the ratio (2.33) determined for the reaction catalyzed by Lohmann's isomerase alone. This indicates that the average value of 17 per cent mannose-6-phosphate for the equilibrium established in the presence of both isomerases cannot deviate much from the true value.

DISCUSSION

The isomerase isolated from muscle which has mannose-6-phosphate as one substrate has been called "phosphomannose isomerase." In order to name Lohmann's isomerase (which catalyzes the equilibrium between glucose-6-phosphate and fructose-6-phosphate) unambiguously according to substrate, the primary product of mannose-6-phosphate isomerization would have to be known.

Although phosphomannose isomerase has not so far been isolated from yeast, its occurrence is indicated by the fact that fructose-1,6-diphosphate is an intermediary product of mannose fermentation by yeast. Lohmann's isomerase, prepared from yeast, does not act on mannose-6-phosphate, the primary phosphorylation product of mannose by yeast hexokinase. A separate isomerase which acts on this product is therefore indicated.

Lohmann's isomerase from muscle has also been prepared free of phosphomannose isomerase. The equilibrium value of 70 per cent glucose-6-phosphate and 30 per cent fructose-6-phosphate obtained with this enzyme preparation was similar to that originally reported by Lohmann (3). He used a crude muscle extract containing both isomerases and distinguished only between aldose and ketose phosphates. The reason why Lohmann's equilibrium ratios were correct was that incubation was carried out by him for only a few seconds, during which time equilibrium was established with either glucose-6- or fructose-6-phosphate as substrate. Phosphomannose isomerase activity in crude muscle extracts is much weaker, so that hardly any mannose-6-phosphate could have been formed in Lohmann's experiments. With longer incubation periods, up to 17 per

cent of mannose-6-phosphate is formed and this would affect the ratio, aldose phosphate to ketose phosphate, as determined by Lohmann.

The author acknowledges with gratitude the suggestions of Dr. C. F. Cori during the course of this work.

SUMMARY

Phosphomannose isomerase has been partially purified from rabbit muscle. In the presence of this enzyme and of Lohmann's isomerase the equilibrium mixture of hexose monophosphates contained 57.4, 25.5, and 17.1 per cent of glucose-, fructose-, and mannose-6-phosphate respectively. The same ratio of glucose- to fructose-6-phosphate as with both isomerases has been obtained when equilibrium was established with a preparation of Lohmann's isomerase which was free of phosphomannose isomerase. The primary product of mannose-6-phosphate isomerization (either glucose- or fructose-6-phosphate) could not be determined because it has not been possible to prepare phosphomannose isomerase completely free from Lohmann's isomerase.

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A COMPARATIVE STUDY OF HEXOKINASE FROM YEAST AND ANIMAL TISSUES*

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Yeast hexokinase catalyzes the transfer of one phosphate group of adenosinetriphosphate (ATP) to fructose, glucose, and mannose, converting them to the respective 6-esters. At concentrations which saturate the enzyme, the order in the rate of phosphorylation is fructose > glucose > mannose. The question has been investigated whether yeast hexokinase has one or more active centers for the utilization of these three hexoses. It was found that from equimolar mixtures of the pairs, fructose-glucose or fructose-mannose, the aldo sugars were much more rapidly removed than the keto sugar. There was also mutual inhibition between the pair mannose-glucose, the former sugar being a stronger inhibitor than the latter. The order of substrate affinity for the enzyme was mannose > glucose > fructose. In the case of fructose it seems probable that it is the furanose form (with a free hydroxyl on carbon 6) which is acted upon by the yeast enzyme.

Brain hexokinase also showed inhibition of fructose utilization by glucose or mannose. Fractionation of the brain enzyme did not result in a change of the relative rates of glucose and fructose utilization and the primary products formed were the respective 6-esters.

It was possible to obtain enzyme preparations from liver and muscle which acted on fructose but not on glucose and vice versa. The fructokinase of liver and muscle was not inhibited by glucose, and the primary reaction product, at least in the case of liver, appeared to be fructose-1-phosphate. Enzymes which act on fructose-1-phosphate have been found in liver and muscle, but not in brain.

Materials and Methods

Crystalline hexokinase was prepared from bakers' yeast as previously described (1). Partially purified brain hexokinase was obtained by

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$(\text{NH}_4)_2\text{SO}_4$ fractionation of aqueous extracts of acetone-dried homogenates of fresh beef or sheep brain cortex (2).

Phosphomannose isomerase was obtained from rabbit muscle in partially purified form by the procedure described in the preceding paper (3). Lohmann's phosphohexose isomerase was partially purified from rabbit muscle extract according to an unpublished method of A. A. Green. *Zwischenferment* was prepared from brewers' yeast by the methods of Warburg and Christian (4) and Negelein and Gerischer (5). It was separated from hexokinase by repeated isoelectric precipitations at pH 4.8 and by allowing solutions to stand for several days at 0°.

The D-glucose used was Mallinckrodt's analytical reagent. The D-fructose (crystalline) and D-mannose were Pfanstiehl's c.p. special grade. ATP was prepared from rabbit muscle by a modification of the method described by Lohmann (6). Triphosphopyridine nucleotide (TPN) was prepared from horse liver according to a modification of the method used by Warburg, Christian, and Griesse for the isolation of TPN from red cells (7).

The rate of the hexokinase reaction was determined in three ways. The first procedure was the direct measurement of sugar disappearance in aliquots of the reaction mixture after precipitation of protein and phosphorylated sugars with $\text{Ba}(\text{OH})_2$ and ZnSO_4 . Reducing sugars were estimated in the filtrates by the method of Nelson (8). Fructose was measured by the procedure of Roe (9). Sugar disappearance was calculated by difference between initial and incubated samples. This method had rather limited usefulness for the determination of enzyme-substrate dissociation constants because a determination of initial rates, especially at low sugar concentrations, required that only a small fraction of the substrate be used up. Hence only a narrow range of sugar concentrations could be investigated.

Two other methods of much greater sensitivity and flexibility were based on the spectrophotometric determination of the reaction product of the hexokinase reaction, hexose-6-phosphate, by means of *Zwischenferment* and TPN.

In the "direct" method all auxiliary enzymes (*Zwischenferment* and one or two isomerases, as needed) were present in excess; the rate of reduction of TPN at 340 $\text{m}\mu$, as observed in the Beckman spectrophotometer, was then a measure of the rate of the hexokinase reaction. Each microgram of sugar phosphorylated in a reaction mixture of 3 ml. in a 1 cm. cell corresponds to a $\log I_0/I$ reading¹ of 0.0115. In some cases discrepan-

¹ The value of 6.22×10^4 sq. cm. per mole for a cuvette of 1 cm. was used as the molecular extinction coefficient for reduced TPN (10).

cies were noted between the rate of hexose utilization as measured spectrophotometrically and by chemical analysis, the former method giving lower values than the latter. This was generally the result of not using a sufficient excess of auxiliary enzymes.

In the "indirect" spectrophotometric method a reaction mixture was prepared in multiples of the following: 0.5 ml. of 0.05 M veronal, pH 8, 0.1 ml. of 0.1 M $MgCl_2$, 0.1 ml. of 0.1 per cent insulin, 0.1 ml. of 0.013 M ATP, 0.3 ml. of H_2O . Samples (1.0 ml.) were transferred to small tubes and 0.1 ml. of hexose solution (of desired concentration) was added. After temperature equilibration at 30° , the reaction was started by the addition of 0.1 ml. of a solution containing 0.05 to 0.3 γ of crystalline yeast hexokinase stabilized with insulin (1). The reaction was stopped by the addition of 0.5 ml. of 0.1 N HCl, an amount sufficient to inactivate the enzyme. After neutralization, an aliquot was transferred to a cuvette, and to this were added 0.5 ml. of 0.05 M veronal, pH 8, 0.2 ml. of *Zwischenferment*, and 0.1 ml. of Lohmann's or phosphomannose isomerase.² The volume was brought to 2.95 ml. and, after determining the initial optical density, with water in the reference cell, 0.05 ml. of TPN (0.3 μM) was added, and the reduction of TPN was followed to the end-point which was usually reached within 10 minutes. Fig. 1 shows a time curve for hexokinase activity obtained by the indirect spectrophotometric method; the rate of the reaction remained constant. Even with the lowest concentrations of sugar or ATP used, experimental conditions could be so arranged with respect to hexokinase concentration and time of incubation that the measurements fell on the linear portion of the rate curve.

Results

Yeast Hexokinase—Dissociation constants obtained with crystalline yeast hexokinase and its substrates are given in Table I. The constants were calculated from linear plots of $1/v$ versus $1/s$, v being the initial rate and s the substrate concentration. The K_s values for glucose and mannose represent the affinity of hexokinase for the α - and β -pyranose forms of these sugars, these being the prevalent forms in which these aldoses exist in solution. Gottschalk (11) and others have shown that the α and β forms of glucose are fermented at the same rate by yeast. This is also true for mannose (12).

² It is essential that these enzymes be free of hexokinase because the solution to be analyzed for hexose monophosphate contains free sugar and ATP. Similarly, phosphofructokinase must be absent; otherwise some of the monophosphate would be diverted from the *Zwischenferment* reaction by conversion to fructose diphosphate. Finally, the *Zwischenferment* preparation must be sufficiently purified so that it does not contain proteins which reoxidize reduced TPN.

D-Fructose exists almost entirely in the β -pyranose and β -furanose forms in aqueous solution (13). By measuring fermentation of an equilibrated fructose solution at pH 4.5 at 0°, conditions favoring minimal mutarotation (13, 14), Gottschalk estimated that 12 per cent of the fructose was present as furanose (15). This estimate was based on his finding that

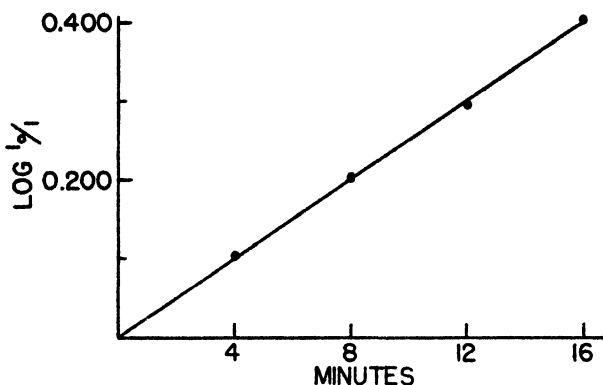


FIG. 1. Glucose phosphorylation by ATP in the presence of crystalline yeast hexokinase. Reaction rate determined by the indirect spectrophotometric method.

TABLE I

Substrate-Enzyme Dissociation Constants for Yeast Hexokinase

Dissociation constants are expressed in moles per liter and represent averages of several determinations obtained by the direct and by the indirect spectrophotometric method. The figures in parentheses indicate the number of determinations made.

Substrate	Dissociation constant (K_s)	
	Direct method	Indirect method
Glucose	1.3×10^{-4} (4)	1.6×10^{-4} (4)
Fructose	1.2×10^{-3} (4)	1.7×10^{-3} (1)
Mannose		1.0×10^{-4} (3)
ATP (glucose)		9.5×10^{-5} (3)
" (fructose)		4.2×10^{-5} (2)

only the furanose form of fructose is fermented by yeast (16-18). The K_s values for fructose in Table I should, therefore, have been calculated for the concentration of β -fructofuranose present at pH 8 at 30°, roughly 20 per cent of the total fructose concentration. The mutarotation equilibrium also depends on the concentration of fructose, and from data available in the literature one cannot arrive at a reliable estimate of the pro-

portion of furanose present at each concentration used. The values in Table I have therefore been calculated for the total concentration of fructose present initially in each case.

Two types of experiments were carried out to test Gottschalk's assumption that the exclusive fermentation of the furanose form of fructose by yeast is due to the specificity of hexokinase for this form. In both cases incubation was at pH 5.9 at 0°, under which conditions the rate of mutarotation of fructose is only about 1.7 times as rapid as the minimal rate found at pH 4 (13). The reaction mixture contained 0.5 ml. of 0.2 M acetate, pH 5.9, 0.1 ml. of 0.2 M MgCl₂, 0.5 ml. of 0.4 per cent hexose, and enzyme in a volume of 2.2 ml. The mixture was chilled in an ice bath for 5 minutes before 1 ml. was added to 0.1 ml. of 0.06 M ATP (or 0.1 ml. of water in the control sample) to start the reaction. After incubation, Ba(OH)₂ and ZnSO₄ were added to control and to experimental samples and the filtrates analyzed for sugar.

TABLE II

Proportionality of Fructose and Glucose Utilization to Hexokinase Concentration under Conditions Giving Low Rates of Mutarotation

The figures indicate micrograms of hexose used during incubation for 12 minutes at pH 5.9, 0°. Approximately 900 γ of hexose were present initially.

Substrate	Hexokinase	
	34 γ	68 γ
Fructose....	250	230
Glucose ...	425	735

In the experiment in Table II an equilibrated solution of glucose or fructose was added (about 900 γ each) and the sugar utilization measured after 12 minutes of incubation at two enzyme concentrations. When glucose was the substrate, the amount of sugar used rose from 425 to 735 γ when the enzyme concentration was doubled. With fructose the utilization amounted to 250 γ and did not increase when the enzyme concentration was doubled. This indicates that fructose utilization was limited by some non-enzymatic factor, presumably the rate of conversion of the pyranose to the furanose form.

In another experiment, the rate of fructose utilization was measured under the above conditions, except that the reaction mixture contained either freshly dissolved crystalline β -D-fructopyranose or an equilibrated solution of fructose. The results given in Fig. 2 show that the initial rate of phosphorylation of freshly dissolved fructopyranose was significantly lower than that of an equilibrated solution of fructose. The ex-

periments may be regarded as a confirmation of a theory originally proposed by Hopkins (19) that yeast ferments the furanose form of fructose.

The K_s values in Table I show that mannose has a higher affinity (smaller dissociation constant) for the enzyme than glucose, while fructose has a lower affinity, even if the K_s values were calculated for what might be a reasonable estimate of the fructofuranose concentration. The dissociation of ATP and yeast hexokinase appears to be affected by the hexose present as phosphate acceptor. This should be verified by additional experiments.

Competitive Inhibition—If hexokinase has only one active center for the utilization of the three hexoses, one would expect that one hexose

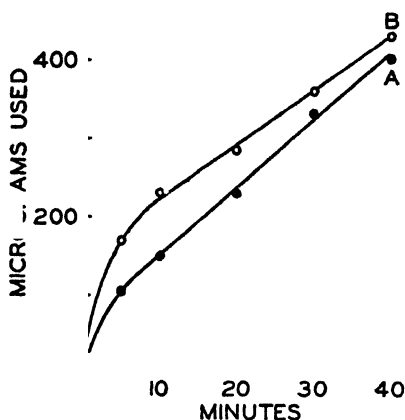


FIG. 2. Comparison of rate of phosphorylation of freshly dissolved β -D-fructopyranose (Curve A) with that of a mutarotated fructose solution (Curve B). Incubations with yeast hexokinase and ATP at pH 5.9, 0°. Initial concentration of fructose 4.5×10^{-3} M.

would inhibit the phosphorylation of another competitively and that the degree of inhibition would be determined by the relative affinities of the three sugars for the enzyme. Accordingly the order of mutual inhibition should be mannose > glucose > fructose.

In Experiments 1 to 3 in Table III the pairs fructose-glucose and fructose-mannose were investigated. The initial concentration of each sugar, alone or in a mixture, was 4.3×10^{-3} M, enough to give 75 per cent saturation of the enzyme with respect to fructose and complete saturation with respect to the two aldoses. From determinations of total reducing power by Nelson's method and of fructose by Roe's method, the disappearance of each sugar from the mixture could be calculated. There was almost complete inhibition of fructose utilization either by glucose or

mannose, while fructose had little effect on the utilization of the two aldose sugars.³

A detailed analysis was carried out for the pair glucose-mannose with the indirect spectrophotometric method which permits initial rates to be measured, so that concentrations of reactants do not change appreciably during the period of measurements. In Fig. 3 the reciprocals of the initial velocity are plotted against the reciprocals of the substrate concentration. The straight lines drawn for glucose alone (Curve A) or mannose alone (Curve B) intercept the ordinate at 7.5 and 10, respectively.⁴ The dissociation constants calculated from these intercepts and the slopes are 1.9×10^{-4} M for glucose and 0.8×10^{-4} M for mannose. Curve C repre-

TABLE III
Mutual Inhibitory Effects of Hexoses (Yeast Hexokinase)

The initial concentration of hexoses was 4.3×10^{-3} M (1800 γ per 2.3 ml. of reaction mixture) and of ATP, 5×10^{-3} M. Incubations were with crystalline yeast hexokinase at 30°, pH 8, except in Experiment 2, where pH was 7.

Experiment No.	Substrates	Fructose used	Glucose or mannose used	Per cent inhibition	
				Fructose	Glucose or mannose
1	Fructose	γ	γ		
	Glucose	800	570		
2	Fructose + glucose	20	570	98	0
	Fructose	885			
3	Glucose		550		
	Fructose + glucose	105	465	88	15
	Fructose	640			
	Mannose		170		
	Fructose + mannose	40	140	94	18

sents inhibition of glucose utilization by mannose and Curve D the reverse. The fact that these curves have intercepts essentially the same as those obtained in the absence of the inhibitory hexose indicates that the inhibition is competitive. When K_i mannose is calculated from Curve C, that is when mannose is considered as an inhibitor of glucose, the value is

³ The following substances, at a concentration of 10^{-3} M, did not inhibit fructose utilization by yeast hexokinase: glucose-1-phosphate, glucose-6-phosphate, mannose-6-phosphate, galactose-1-phosphate, galactose.

⁴ This indicates that the ratio of mannose to glucose utilization is 0.75 at complete saturation with substrate. Considerably lower ratios were obtained previously with a manometric method (1), perhaps because at the high concentration used (0.03 M) some impurity present in the mannose sample exerted an inhibitory effect of the enzyme.

very nearly the same as K_s mannose, 0.77 and 0.8×10^{-4} M, respectively. The corresponding values obtained for glucose are 2.0 and 1.9×10^{-4} M, respectively.

In the experiment in Fig. 3 each sugar in the mixture is both a substrate and an inhibitor, and the case may therefore be treated as one of competitive inhibition (as in Fig. 3) or as one of substrate competition (as in Table IV). In the former case, v , the rate of disappearance of only one of the two sugars from the mixture enters the equation, while the other sugar is considered as inhibitor; *e.g.*, $1/v = 1/V + 1/V(K_s + K_s I/K_i) 1/s$.

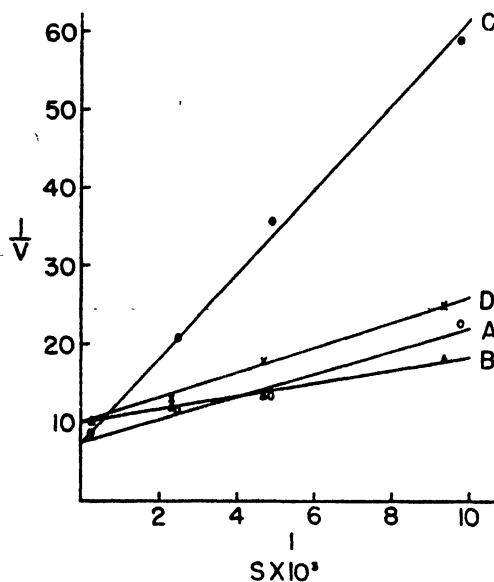


FIG. 3. Competitive inhibition of glucose phosphorylation produced by mannose and vice versa. Curve A, glucose alone; Curve B, mannose alone; Curve C, inhibition of glucose by 2×10^{-4} M mannose; Curve D, inhibition of mannose by 2×10^{-4} M glucose. The indirect spectrophotometric method was used.

In the latter case the equation (11) contains v_g and v_m , the rates of disappearance of both glucose and mannose from the mixture, *e.g.* $v_g/v_m = G/M \cdot V_g/V_m \cdot K_{s,m}/K_{s,g}$, where V_g and V_m are the reciprocals of ordinate intercepts in Fig. 3, G and M the respective sugar concentrations, and $K_{s,m}$ and $K_{s,g}$ the respective substrate-enzyme dissociation constants. From the data in Table IV it may be calculated that, on an average, the ratio $K_{s,m}:K_{s,g}$ is 0.40 ; this is in agreement, within experimental error, with the ratios calculated from the experiment in Fig. 3, but is lower than the ratio found in Table I. It is possible that the ATP concentration has an effect on the

sugar-enzyme dissociation constant; this had not been taken into consideration when these experiments were carried out. The possibility that mannose-6-phosphate inhibits the phosphorylation of glucose has been tested with negative results.

Brain Hexokinase—This enzyme utilizes fructose, glucose, and mannose at about the same relative rates as does yeast hexokinase. The primary product of glucose phosphorylation has been shown to be glucose-6-phosphate (2). The experiment in Fig. 4 indicates that the corresponding product with fructose is fructose-6-phosphate and not (as seemed possible) fructose-1-phosphate. Curve A shows that a system containing *Zwischenferment*, purified Lohmann's isomerase, and brain hexokinase was unable to catalyze the conversion of fructose-1-phosphate to fructose-6-phos-

TABLE IV

Glucose and Mannose As Competitive Substrates for Hexokinase

The same experiment as in Fig. 3, including two additional pairs of glucose-mannose. The concentration of glucose and mannose in the mixture is given in moles per liter. v glucose and v mannose correspond to the initial rate of disappearance of the two sugars from the mixture in terms of $\log I_0/I$ readings. $K_{s,m}:K_{s,g}$, the ratio of substrate-enzyme dissociation constants, calculated from the equation given in the text.

Glucose ($M \times 10^3$)	0.102	0.205	0.410	0.205	0.205
Mannose ($M \times 10^3$)	0.214	0.214	0.214	0.107	0.428
v glucose	0.017	0.028	0.048	0.042	0.019
v mannose	0.067	0.056	0.046	0.040*	0.077
G:M	0.48	0.96	1.92	1.92	0.48
$v_g:v_m$	0.254	0.50	1.045	1.05	0.247
$K_{s,m}:K_{s,g}$	0.398	0.391	0.410	0.412	0.388

* Calculated, assuming the same ratio, $v_g:v_m$, as in the preceding column. The experimental value obtained (0.047) appears to be erroneous.

phate. That the system was otherwise complete is shown by the effect of addition of fructose-6-phosphate at 11.5 minutes; a rapid reduction of TPN occurred. Curve B represents the results obtained with the same system of enzymes as in Curve A, except that ATP was also present. Addition of fructose at 5.5 minutes resulted in a rapid reduction of TPN, showing that fructose had been converted to fructose-6-phosphate by brain hexokinase. That the brain fraction was the only significant source of hexokinase activity is shown by Curve C. In this case, the brain fraction was omitted initially and the addition of fructose at 5.5 minutes had no effect. When the brain fraction was added at 11.5 minutes, the rate of reduction of TPN proceeded at a rate comparable to that observed in Curve B.

The formation of fructose-6-phosphate by brain hexokinase would in-

dicates that it also acts on the furanose form of fructose. Dissociation constants for glucose and fructose could not be determined accurately by the chemical method. Judging from the experiments of Meyerhof and Wilson (20), glucose has a higher affinity for brain hexokinase than fructose. They found that glucose and fructose were utilized at about the same rate when the initial sugar concentration was 2×10^{-2} M. At one-tenth this concentration no decrease in the rate of glucose utilization occurred, while fructose was utilized at about one-half the rate of glucose.

A further similarity between yeast and brain hexokinase is that glucose and mannose have a strong inhibitory effect on the phosphorylation of

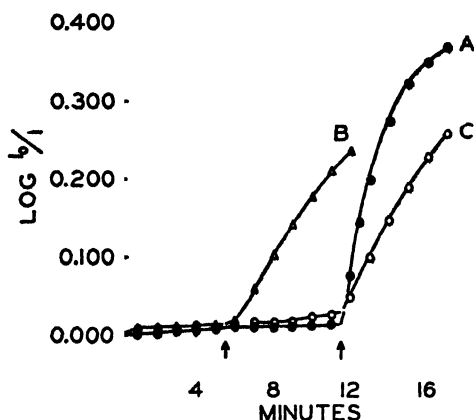


FIG. 4. Demonstration that fructose-6-phosphate is the primary product of fructose phosphorylation by ATP in the presence of sheep brain acetone powder extract. Curve A, *Zwischenferment* plus Lohmann's isomerase and brain fraction present initially (no ATP); fructose-1-phosphate ($2 \mu\text{M}$) added at 5.5 minutes, fructose-6-phosphate ($1 \mu\text{M}$) at 11.5 minutes. Curve B, same as Curve A plus ATP; fructose added at 5.5 minutes. Curve C, same as Curve B, but without brain fraction initially; fructose added at 5.5 minutes, brain fraction at 11.5 minutes.

fructose by the brain enzyme. In the experiments in Table V the utilization of fructose from equimolar mixtures of glucose and fructose was completely suppressed, while that of glucose was only slightly diminished. Only when the initial concentration of fructose was 4 times that of glucose was there some utilization of fructose, amounting to 15 per cent of that found with the same concentration of fructose alone.⁵ As with yeast

⁵ In this experiment the initial glucose concentration was 20 mg. per cent and decreased to about half that value during incubation. The well known observation that fructose, in contrast to mannose, is ineffective in causing an immediate relief of hypoglycemic shock may be explained by the fact that glucose is a much stronger competitive inhibitor of fructose than of mannose utilization by brain hexokinase.

hexokinase, the product of glucose phosphorylation, glucose-6-phosphate, did not inhibit the phosphorylation of fructose by brain hexokinase.

In the experiments summarized in Table VI the ratio of fructose to glucose utilization in crude brain extract was the same as that obtained with a purified ammonium sulfate fraction of the enzyme. Fractionation with acetone also failed to cause a shift in the ratio. (As shown in the next section, these were the procedures which led to a separation of fructokinase and glucokinase from muscle.) Somewhat lower F:G ratios

TABLE V
Mutual Inhibition of Glucose, Mannose, and Fructose Phosphorylation
(Brain Hexokinase)

2.3 ml. of reaction mixtures incubated at 30° in 0.03 M veronal buffer, pH 8. Each contained 7 μ M of ATP, 20 μ M of MgCl₂, and 3 mg. of sheep brain protein obtained between 0.3 and 0.5 saturation with (NH₄)₂SO₄. Different enzyme preparations were used in all but the first two experiments.

Incubation period min.	Initial concentration, M $\times 10^4$		Fructose used γ	Glucose used γ	Per cent inhibition	
	Fructose	Glucose			Fructose	Glucose
20	4.33	0	660			
	0	4.83		410		
	4.33	4.83	0	315	100	23
20	2.11	0	520			
	0	2.38		375		
	2.11	2.38	0	360	100	4
15	1.06	0	290			
	0	1.18		245		
	1.06	1.18	0	210	100	14
15	4.25		380			
		1.15		225		
	4.25	1.15	55	185	85	18
20	4.5		615			
	4.5	4.5*	5		100	

* Mannose.

were obtained with isoelectric precipitates of the brain enzyme. The results as a whole indicate that the predominant type of hexokinase found in an extract of acetone-dried brain is of the yeast type.

Muscle Hexokinase—Crude rabbit muscle extracts are able to phosphorylate fructose and glucose. When such extracts are fractionated with (NH₄)₂SO₄, the former activity can be separated from the latter. Extracts of rat muscle show a more rapid glucose than fructose utilization (F:G ratio about 0.1) and the enzyme which acts on glucose can be concentrated by precipitation with acetone.

The crude extract used for the experiments in Table VII was prepared by extracting ground rabbit muscle twice with 1 volume of cold 0.03 M KOH and once with 0.5 volume of distilled water. The filtrates obtained

TABLE VI
Ratio of Fructose to Glucose Utilization with Various Fractions of Sheep Brain Hexokinase

An aqueous extract of acetone-dried brain was the "crude" fraction; others were prepared from this extract. The initial concentrations of sugar (glucose or fructose) and ATP were 4×10^{-3} M. Incubated in 0.05 M veronal buffer at pH 8 at 30°.

Fraction	No. of experiments	Ratio of utilization of fructose to glucose
Crude...	7	1.5
Dialyzed crude...	3	1.5
20% acetone...	1	1.5
25-40% acetone...	1	1.5
Ppt., pH 5.2...	3	1.1
Supernatant fluid of ppt., pH 5.2...	1	1.4
Ppt., 0.3-0.5 saturation $(\text{NH}_4)_2\text{SO}_4$...	4	1.5

TABLE VII
Utilization of Fructose and Glucose by Rabbit Muscle Extracts

The initial concentrations of sugar (glucose or fructose) and ATP were 3×10^{-3} M; MgCl_2 was 8×10^{-3} M. Fluoride (0.06 M) was present in the reaction mixture with crude extracts. Incubations were in 0.04 M veronal buffer, pH 8. The figures represent micromoles of sugar used per gm. of protein per 30 minutes at 30°.

Experiment No.	Crude extract		0.41-0.5 saturated $(\text{NH}_4)_2\text{SO}_4$ fraction		Protein per 100 gm. muscle	
	Fructose	Glucose	Fructose	Glucose	Crude extract	$(\text{NH}_4)_2\text{SO}_4$ fraction
					gm.	gm.
1	36	37	262	8	2.05	0.170
2	32	35	929	0	3.04	0.076
3	25	83	591	45	2.22	0.101
4	30	86	694	0	2.25	0.169
5	4	69	694	0	2.12	0.149

by straining the muscle suspension through gauze were combined (crude extract) and treated with saturated $(\text{NH}_4)_2\text{SO}_4$ adjusted to pH 7.8. The precipitate which formed at 0.41 saturation was filtered off in the cold and discarded. To the filtrate was added $(\text{NH}_4)_2\text{SO}_4$ solution to 0.5 sat-

uration, and, after thorough chilling in an ice bath, the precipitate was collected by centrifugation. For activity tests or further fractionation, it was dissolved in 0.1 M veronal buffer at pH 8. The 0.41 to 0.5 fraction (2 to 8 per cent of the protein of the crude extract) showed a 10 to 30 times greater activity toward fructose than the crude extract but had practically no ability to utilize glucose. This indicates that two hexokinases are involved in the phosphorylation of fructose and glucose in rabbit muscle. Both enzymes are activated by Mg^{++} ions.

Rat muscle was minced with scissors on a glass plate in the cold and extracted for about 5 minutes in a mortar (without abrasive) with 1.5 volumes of cold distilled water. The pulp was strained through gauze and the filtrate centrifuged for about 5 minutes at about 7000 r.p.m. For

TABLE VIII

Utilization of Glucose and Fructose by Fractions from Rat Muscle Extract

The figures indicate micromoles of sugar used per gm. of protein in 30 minutes at 30°. Initial hexose concentration about 3.4×10^{-3} M, except in Experiment 2 in which the concentration was half this amount. Fluoride (0.06 M) was present in measurements with crude extracts. Incubated in 0.04 M veronal buffer, pH 8.

Experiment No.	Fraction	Fructose	Glucose	F:G
1	Crude	46	351	0.13
	30% acetone	79	652	0.12
2	Crude	4	130	0.02
	30% acetone	43	240	0.18
	45% "	10	184	0.05
	51% "	7	42	0.17
3	Crude	106	400	0.26
	0.35-0.54 saturation $(NH_4)_2SO_4$	133	278	0.48

fractionation with cold acetone the extract was adjusted to pH 6.0 with dilute HCl, a method originally used by S. P. Colowick. The results in Table VIII show that in some cases (Experiment 2) the crude extract itself had practically no ability to phosphorylate fructose. Treatment with acetone, although it effected some purification, did not eliminate the residual activity toward fructose. Fractionation with $(NH_4)_2SO_4$ (Experiment 3) led to a concentration of fructose activity (F:G ratio of 0.48). The experiments favor the assumption that separate kinases for glucose and fructose are also present in rat muscle. The concentration of the former enzyme in rat muscle extract is about 5 times greater than in rabbit muscle extract.

The experiments in Table IX show that the 0.41 to 0.5 fraction of rabbit

muscle and subfractions prepared from it contain enzymes which remove added fructose-1-phosphate and fructose-6-phosphate in the presence of ATP. In both cases the product formed was fructose-1,6-diphosphate, which was determined quantitatively in a test system consisting of aldolase, triose isomerase, triose phosphate dehydrogenase, DPN, and arsenate (21). In all fractions the activity of these two enzymes was so much greater than that of fructokinase that the primary phosphorylation product of fructose (either fructose-1- or fructose-6-phosphate) could not be established.

TABLE IX

Effect of Fractionation with $(\text{NH}_4)_2\text{SO}_4$ on Utilization of Substrates by Rabbit Muscle Proteins

The reaction mixtures consisted of 3.5×10^{-3} M fructose (F), fructose-1-phosphate (F1P), or fructose-6-phosphate (F6P), 5×10^{-3} M ATP, 10×10^{-3} M MgCl_2 in 0.04 M veronal buffer, pH 8. The methods of analysis for F1P and F6P in neutralized trichloroacetic acid filtrates are described in the text.

Experiment No.	Fraction		Sugar used, μM per mg. protein per 30 min. at 30°			Ratios		
			F	F1P	F6P	F1P:F	F6P:F1P	F6P:F
1	Original	41-50	0.696	6.66	72.6	9.6	10.9	104
	Subfraction	0-40	0.913	10.9	50.4	11.9	4.6	55
	"	40-45	0.582	6.42	85.2	11.0	13.3	146
	"	45-50	0.072	0.76	14.9	10.6	19.5	207
2	Original	41-50	0.738	5.28	75.6	7.2	14.3	102
	Subfraction	0-40	1.27	10.6	108	8.3	10.2	85
	"	40-45	0.432	1.24	70.8	2.9	57.0	164
3	"	0-39	0.828	0.72	89.4	8.1	13.3	108
	"	38-42	1.21	9.66	136	8.0	14.1	112
	"	42-46	0.546	4.94	97.2	9.0	19.7	178

The conversion of fructose-1-phosphate to fructose-1,6-diphosphate in muscle fractions occurs only in the presence of ATP.⁶ In Table IX the ratio of utilization, F6P:F1P, in the different fractions varied from about

⁶ It seemed possible that a mutase type of reaction was involved, fructose-1-phosphate \rightarrow fructose-6-phosphate, followed by fructose-6-phosphate + ATP \rightarrow fructose-1,6-diphosphate. In such a reaction fructose-1,6-diphosphate might be expected to act as coenzyme, in analogy to the coenzyme effect of glucose-1,6-diphosphate in the phosphoglucomutase reaction (22, 23). However, no disappearance of fructose-1-phosphate could be detected when ATP was omitted and fructose-1,6-diphosphate was added. The fructose-1-phosphate was synthesized according to Raymond and Levene (24).

5 to 57, which suggests that two different enzymes are involved. In order to distinguish them they might be referred to as 1-phospho- and 6-phosphofructokinase. Brain extracts containing the latter enzyme cannot form fructose-1,6-diphosphate from fructose-1-phosphate and ATP, which also speaks for the distinct nature of the two enzymes.

There are two other features which distinguish the muscle fructokinase from the hexokinase of brain or yeast. One is that glucose does not inhibit the phosphorylation of fructose by muscle fructokinase when the hexoses are added in equimolar concentration (about 4×10^{-3} M). The other is that fructokinase is apparently saturated by its substrate only at unusually high concentrations, if at all, since the straight line obtained by

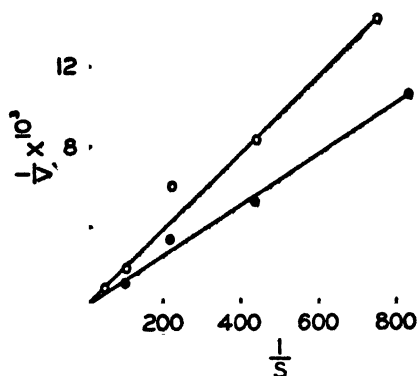


FIG. 5. Two experiments in which increasing fructose concentrations appear unable to saturate a preparation of rabbit muscle fructokinase. s , the substrate concentration, is given in moles per liter.

plotting $1/v$ against $1/s$ goes through the origin. This is illustrated in Fig. 5.

Liver Hexokinase—Ochoa, Cori, and Cori (25) mentioned briefly the isolation of fructose-1-phosphate from rat liver homogenates which had been incubated aerobically with fructose, an oxidizable substrate (glutamate), Mg^{++} , NaF, and a catalytic amount of ATP. The ester was characterized by its specific rotation and by its rate of hydrolysis in acid, both of which distinguish it sharply from other known phosphate esters. They also reported experiments which showed that, when fluoride was omitted, fructose was converted to glucose (26). Addition of fructose-1-phosphate to liver homogenates in the absence of fluoride also gave rise to the formation of glucose.

It has since been found that liver contains a mutase system for the conversion of fructose-1-phosphate to hexose-6-phosphate. The formation

of fructose-1-phosphate has been demonstrated in rat liver homogenates under anaerobic conditions with ATP as phosphate donor, and the enzyme which catalyzes this reaction has been characterized further. It is a fructokinase which resembles that found in muscle, since it does not act on glucose and since its activity is not inhibited by glucose. Finally, liver contains a glucokinase, as shown in Table X. The activity of this enzyme cannot be measured accurately in liver homogenates because of the presence of glucose-6-phosphatase which is incompletely inhibited by fluoride. Another source of error is the formation of maltose and other

TABLE X

Phosphorylation of Fructose and Glucose by Rat Liver Homogenate

Incubated for 20 minutes at 30° in the presence of 0.06 M NaF, 4×10^{-3} M hexose, 5×10^{-3} M ATP, and 8×10^{-3} M MgCl₂ in potassium-phosphate buffer, pH 7.6. The figures represent micrograms of hexose utilized per 2 to 2.5 ml. of reaction mixture containing 1 ml. of homogenate (equivalent to 200 mg. of liver). The livers of fasted rats were perfused with salt solution prior to homogenization in 0.9 per cent sodium chloride solution.

Experiment No.	Fructose	Glucose
1	230	65
2	375	0
3	320	25
4	375	100
5		140
6*	0	75
7*	0	102
8†	0	125
9†	55	150

* 0.25 to 0.45 fraction with (NH₄)₂SO₄; fructose used before fractionation, 130 and 250 γ, respectively.

† Rabbit.

reducing substances from glycogen by diastatic activity. As has been pointed out previously (27), diastatic activity is particularly marked in rat liver and cannot be completely eliminated by previous perfusion of the liver. For these reasons the values recorded in Table X are minimal values for glucokinase activity. Experimental details concerning these findings will be reported in a subsequent paper.

SUMMARY

1. Substrate-enzyme dissociation constants were determined for crystalline yeast hexokinase with a spectrophotometric method. The order of

affinity for the enzyme was mannose > glucose > fructose, while the order of maximal velocity, on saturation with substrate, was fructose > glucose > mannose. Evidence was presented that the enzyme reacts with that form of fructose in solution which contains a free hydroxyl group on carbon 6 (furanose form, roughly 20 per cent of total fructose concentration in these experiments). The ATP-enzyme dissociation constant was found to vary with the type of hexose present as phosphate acceptor.

2. With brain and yeast hexokinase there was nearly 100 per cent inhibition of fructose utilization by an equimolar concentration of either glucose or mannose, while fructose had little inhibitory effect on the utilization of the two aldo sugars. There was also mutual inhibition between the pair mannose-glucose, the former sugar being a stronger inhibitor than the latter. From the fact that K_i mannose was the same as K_i mannose and K_i glucose the same as K_i glucose, it was concluded that they act as substrates and inhibitors at the same center of the enzyme.

3. The first product of fructose phosphorylation with brain hexokinase was shown to be fructose-6-phosphate. Various fractionation procedures did not result in a significant change in the ratio of fructose to glucose utilization. It appears that the predominant type of hexokinase in brain is of the yeast type.

4. The fructokinase of liver and muscle which does not act on glucose is not inhibited by this sugar. Enzymes which act on fructose-1-phosphate (probably the first phosphorylation product of fructose by liver fructokinase) have been found in liver and muscle but not in brain. Liver and muscle were also shown to contain a glucokinase.

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ACETYLCHOLINESTERASE

X. MECHANISM OF THE CATALYSIS OF ACYLATION REACTIONS*

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Ester synthesis catalyzed by esterases has been the subject of many investigations (1, 2). The reversal of acetylcholine hydrolysis by serum esterase has been described by Abderhalden and Paffrath (3) and Ammon and Kwiatkowski (4). The availability of a highly active and purified acetylcholinesterase and the development of a chemical method for the determination of acetylcholine in the presence of excess of acetate and choline made possible a quantitative analysis of the equilibrium (5). In a preceding paper it has been described that this equilibrium shifts markedly in favor of esterification of choline and acetate with decreasing pH (6). Acethydroxamic acid formation was also found to be catalyzed by the enzyme. The optimum for this reaction was found to be pH 6.3.

The picture of the active surface of acetylcholinesterase developed in a series of papers (7-10) suggests that the enzyme contains a basic group capable of reacting with the electrophilic carbon of the carbonyl group of acetylcholine. This reaction occurs similarly with other compounds which are either substrates or inhibitors. The stronger the electrophilic character of the carbon, the greater the reaction with the enzyme. This was observed with a series of derivatives of nicotinic acid in which the electrophilic character of the carbonyl carbon was progressively increased while other essential structural features remained unaltered. The experiments suggest that for the catalysis of formation of choline esters and of hydroxamic acid by acetylcholinesterase an ester should be a more favorable substrate than the corresponding carboxylate ion. This hypothesis implies that during the hydrolysis of the ester the same, or a similar, intermediary is formed as that which occurs between acid and enzyme. Experiments will be described which test this hypothesis.

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Methods

Preparation of Enzyme—440 gm. of electric organ of *Gymnotorpedo occidentalis* (Storer), which had been stored under toluene for 3 years, were homogenized in a Waring blender with 5 per cent ammonium sulfate solution and repeatedly extracted until the total volume of extract was 2660 ml. Sufficient ammonium sulfate was added to raise the concentration to 14 per cent. The precipitate which formed overnight was discarded. Protein was precipitated from the supernatant solution by adding ammonium sulfate until the concentration reached 36 per cent. The precipitate so obtained was redissolved by adding 180 ml. of H_2O . Sufficient ammonium sulfate was added to raise the concentration to 15 per cent; the mixture was centrifuged and the precipitate discarded. Ammonium sulfate was added to the supernatant until the concentration reached 30 per cent. The mixture was centrifuged, the supernatant discarded, and 36 ml. of water were added to the precipitate. The 40 ml. of solution so obtained were dialyzed against 0.025 M phosphate buffer. The resulting enzyme solution hydrolyzed about 4.6 gm. of acetylcholine per ml. per hour and contained about 1.8 mg. of protein per ml.

Determination of Hydroxamic Acid and Choline Esters—The hydroxamic acid formed was determined colorimetrically by the addition of HCl and ferric chloride as described previously (11). The chemical determination of the choline ester formed from monobutyrin and ethyl acetate is difficult, since the amount of the choline ester is small compared with the relatively large amount of the starting esters. Therefore, the frog rectus abdominis test for acetyl- and butyrylcholine has been used.

Results

Hydroxamic Acid Formation—Table I summarizes a few typical experiments in which hydroxamic acid formation from acids and esters at different pH levels was studied. The figures show that the formation from esters is much faster than from acids. The pH optimum for the acids was found to be consistent with the value 6.3 reported previously. The rate of hydroxamic acid formation in one experiment at pH 7.0 and in several experiments at pH 7.5 was low. These figures are not included in Table I. The data presented in Table I are the results of one experiment, whereas the other results were obtained with a slightly different enzyme preparation. The rate of formation at pH 5.5 was higher than that reported in the preceding paper (6). Propionylhydroxamic acid formation showed a similar pH dependence. In contrast to the acids, the hydroxamic acid formation from esters increased rapidly with increasing pH. At 8.0 or higher, hydroxamic acid formation is difficult to measure, since at this pH level

non-enzymatic hydroxamic acid formation is very rapid. At pH 7.5, non-enzymatic hydroxamic acid formation was about 10 per cent of the enzyme-catalyzed synthesis. In evaluating the results, it may be noted that the concentrations of the various esters were not identical because of their limited solubility in water and were markedly lower than the concentrations of the acids.

TABLE I

Hydroxamic Acid Formation from Acids and Esters Catalyzed by Acetylcholinesterase at Various pH Levels

The incubation mixture contained, per ml., 0.25 ml. of enzyme, prepared from electric tissue of torpedo. The enzyme solution contained about 1.8 mg. of protein per ml. and hydrolyzed about 4.6 gm. of acetylcholine per ml. per hour. A control was always run without enzyme. The hydroxylamine concentration was 0.6 M. Phosphate buffer was used for adjusting the pH. At the end of the incubation period, HCl and ferric chloride were added for the colorimetric determination. In the second experiment with ethyl acetate, the enzyme was slightly more diluted than in the others.

Substrate	Concentration	Incubation	Acetylhydroxamic acid formed per ml.			
			pH 5.5	pH 6.5	pH 7.5	pH 8.0
	M	min.	μ M	μ M	μ M	μ M
Sodium acetate	0.5	60	1.54	2.47		0.4
		120	2.62	4.10		0.0
“ propionate	0.5	60	0.66	0.77		0.4
		120	0.99	1.39		0.1
“ butyrate	0.5	60		0.05		
		120		0.05		
Ethyl acetate	0.17	15		10.4	16.0	
		30		14.5	21.4	
		60		17.5	25.3	
“ “		3	0.3	1.4	3.0	
		6	0.7	2.8	6.2	
“ propionate	0.05	60		2.02	2.63	
		120		2.91	3.66	

The rate of formation is roughly proportional to the substrate concentration, as may be seen from the figures in Table II. When the differences in concentration are taken into account, the rate of hydroxamic acid formation is of the order of 100 to 150 times as rapid with an ester as with the corresponding acid at pH 6.5, which is the optimum for the acid but not for the ester. Since the ester reacts at pH 7.5 about twice as rapidly, the difference of the maximum rate is still greater.

The reaction velocity decreases rapidly with time when esters are the substrates, as is seen in Table I. This must be attributed to the rapid

enzymatic hydrolysis of the ester which proceeds simultaneously with the slower hydroxamic acid formation. Therefore, for quantitative evaluation relatively short reaction times must be selected. For the short periods of reaction used in the experiments of Table II the decrease in velocity at the second reading is apparent but not marked. For periods of 3 to 6 minutes (see Table I) the rate was constant. The decrease in reaction velocity is much less when sodium acetate is the substrate.

Monobutyrin also reacts quite readily though less rapidly than ethyl acetate or ethyl propionate, but the non-enzymatic reaction is also quite high. Thus, whereas the non-enzymatic hydrolysis of the butyryl ester is

TABLE II

Hydroxamic Acid Formation As Function of Substrate Concentration

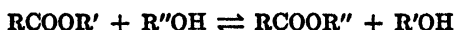
The reaction times were varied in order to get the lowest but still accurate value of hydroxamic acid formed in the incubation mixture. The enzyme was diluted four times for the reaction with ethyl acetate; therefore, the values obtained have been multiplied by 4. 0.025 M phosphate buffer at 6.5 was used. The ionic strength was kept constant by compensating with NaCl when required. The hydroxylamine concentration was 0.6 M; $T = 23^\circ$.

Ethyl acetate			Sodium acetate		
Concentration	Reaction time	Formed per ml. per hr.	Concentration	Reaction time	Formed per ml. per hr.
M	min.	μM	M	min.	μM
0.17	6	71.2	0.8	30	2.20
	12	57.2		60	2.20
0.13	9	47.6	0.6	45	1.95
	19	40.8		90	1.80
0.085	20	26.8	0.4	60	1.30
	28	26.8		120	1.30
0.043	24	12.0	0.2	90	0.80
	30	10.8		180	0.75

much higher than that of the acetyl or the propionyl ester, the enzymatic synthesis decreases rapidly in the order acetate > propionate > butyrate; *i.e.*, in the same order as is observed in the hydrolysis of choline esters. Acetamide, *n*-butyramide, and nicotinamide were also tested. No enzymatic reaction was observed under the experimental conditions. Non-enzymatically, however, the amides react at pH 6.5 with hydroxylamine. This reaction will be analyzed in a separate paper.

Transesterification—From the above results it became evident that in any synthesis catalyzed by acetylcholinesterase the ester should be superior to the corresponding acid. When this principle is applied to the esterifica-

tion of choline with acetate, it may be expected that the enzyme will catalyze transesterification reactions of the type



However, such a synthesis is complicated by the fact that the enzyme will simultaneously split both the substrate ester and the product ester. A steady state will be reached in which the rate of formation equals that of hydrolysis of the new ester. The concentration at the steady state may

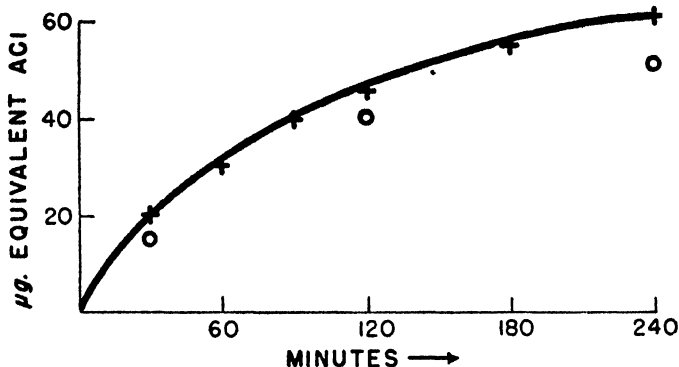


FIG. 1. Butyrylcholine formation from monobutyrin and choline catalyzed by acetylcholinesterase. The composition of the incubation mixture per ml. was as follows: 0.25 ml. of enzyme, 0.4 M monobutyrin, and 0.1 M choline chloride. Phosphate buffer 0.05 M was used at a pH of 7; T 23°. The product formed was tested on the frog's rectus preparation against acetylcholine as the standard. Since a solution of butyrylcholine is about 20 to 25 per cent less active than an equimolecular solution of acetylcholine, the figures indicated on the ordinate should be multiplied by about 1.2 for obtaining the correct value of butyrylcholine formed per ml. All the components of the test mixture alone or in combination with the enzyme were tested on the muscle and did not show any activity in the dilution used for the enzymatic synthesis.

persist for some time, but will eventually decline, due to the decrease in concentration of starting ester.

Fig. 1 represents the results of two experiments in which monobutyrin and choline were incubated with the enzyme. The steady state seems to be reached in about 3 to 4 hours. The usual controls, run simultaneously, included one containing sodium butyrate instead of the ester. No butyrylcholine formation was measurable under the experimental conditions in any of the controls.

It has been previously described that one of the characteristic features of acetylcholinesterase which distinguishes this enzyme from other esterases

is the decrease of the rate of hydrolysis with increasing length of the acyl chain (12, 13). In the case of torpedo enzyme, propionylcholine was described as being hydrolyzed under the experimental conditions at a rate about one-third of that of acetylcholine. Butyrylcholine was not measurably hydrolyzed under the same conditions (14). This, of course, does not indicate absolute inability of the enzyme to attack butyrylcholine. It would be surprising if the enzyme were completely unable to attack this ester. We have studied this question and have found that the concentration of enzyme must be 140 times as high as for acetylcholine in order to split butyrylcholine at the same rate.

In an experiment in which ethyl acetate and choline were used as substrates the steady state was reached much faster than with monobutyryn, in about 10 minutes, even though only one-twelfth of the enzyme concentration was used. The maximum value, however, was lower; 5.5 γ of acetylcholine were formed per ml. of incubation mixture in 5 minutes and 7 to 8 γ in 10, 20, and 40 minutes. After 80 minutes the concentration had declined to 4.5 γ . However, in this case the concentrations of choline and ester were much lower, 0.03 and 0.28 M respectively. The steady state is determined by the rate of formation which depends upon the concentrations of the enzyme and the two substrates, and by the rate of hydrolysis of ester formed which depends upon the concentration of the enzyme and this ester. The concentration of enzyme is of course the same for the two opposing reactions and will therefore not determine the level of the steady state. The concentration of ethyl acetate and choline was much lower than in the experiments presented in Fig. 1. Therefore, the steady state was reached at a lower level, but the difference of the two levels was of the order of magnitude which would be expected from the difference of the substrate concentrations.

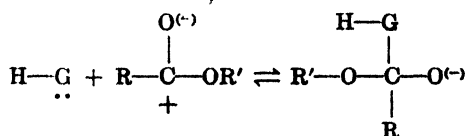
DISCUSSION

As anticipated, the rate of formation of enzyme-catalyzed hydroxamic acid is some 100 times more rapid with esters than with the corresponding carboxylate ions. While the carbonyl carbon of carboxylate ion has little electrophilic character, the carbon of undissociated acid is markedly electrophilic. In accordance with the mechanism proposed (9), it would be expected that the actual reactant is the undissociated carboxylic acid and that the rate should be about the same as with the ester. At pH 6.5 there is only about 1 per cent undissociated acid. Since the rate is approximately proportional to the concentration of the reactant, ester and undissociated acid are about equally effective.

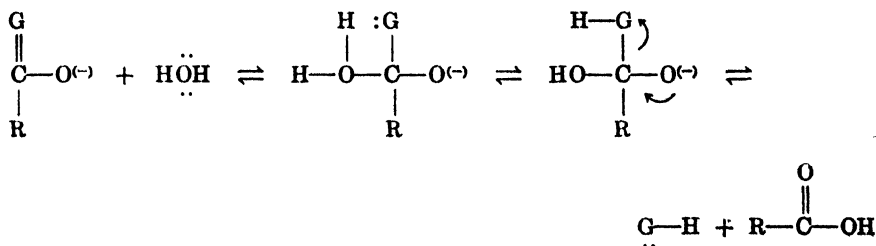
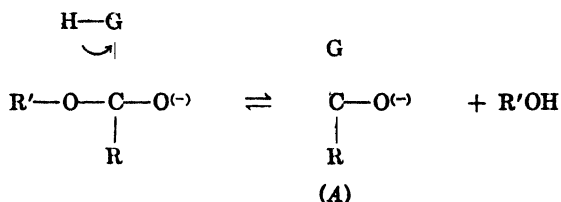
Apparently during the enzymatic hydrolysis of an ester an intermediary is formed which may react with either water (or its ions) or with hy-

droxylamine or choline. Any reaction with H^+ or OH^- is not rate-controlling. Reaction with water results in hydrolysis, while reaction with hydroxylamine produces hydroxamic acid, and with choline it produces choline esters. The relative amounts of the different products depend upon the relative concentrations of these reactants.

The mechanism of enzyme interaction may be outlined as follows:



where $\text{G}-\text{H}$ is that part of the active enzyme surface which contains a basic group, represented by the pair of unshared electrons, and an acid group, represented by the hydrogen atom. The enzyme-substrate complex as shown here is the fundamental structure. The actual structure may be one of a number of more probable minor variations, containing protons or hydrogen bonds joined to one or both oxygens. *A priori* there appear to be several similar mechanisms for the reaction of the complex with nucleophilic reagents. One possible mechanism is the internal elimination of $\text{R}'\text{OH}$.



The same intermediate (A) can be formed from the acid molecule as from the ester. This intermediate can react with water (as shown) or with hydroxyl ion, hydroxylamine, or choline (or with other similar nucleophilic reagents). The formation of the intermediate is the rate-controlling step.

The dependence of the velocity upon pH and reactant concentration can

be developed mathematically if we take into account the acidic dissociation of all reactants including the enzyme. The method of the stationary state along with the experimental observation that the velocity is approximately proportional to the concentration of the reactants yields

$$\frac{K(S)(B)}{K_{EH_2^+} + 1 + \frac{K_{EH^+}}{H^+}}$$

where v is velocity, S may be either acid or ester, and B may be H_2O , OH^- , H_2NOH , or choline. $K_{EH_2^+}$ and K_{EH^+} are dissociation constants for the enzyme. (S) is the concentration of the actual reactant and therefore the stoichiometric concentration $(S)^0$ of an ester but the undissociated acid concentration for the salt of a carboxylic acid. (B) is the stoichiometric concentration $(B)^0$ for choline but the base concentration for hydroxylamine. Thus

$$(S) = (S)^0 \frac{H^+}{K_a + (H^+)}$$

$$(B) = (B)^0 \frac{K_{B^+}}{K_{B^+} + (H^+)}$$

where K_a is the acid dissociation constant for the fatty acid and K_{B^+} is the acid dissociation constant of $(NH_3OH)^+$. For carboxylic acid $K_a \cong 2 \times 10^{-5}$, for hydroxylamine $K_{B^+} \cong 8 \times 10^{-7}$ and $K_{EH_2^+} \cong 7 \times 10^{-7}$, $K_{EH} \cong 5 \times 10^{-10}$. For the reaction of acid plus hydroxylamine the calculated velocity is a maximum at about pH 6.5, as in fact is found experimentally.

For ester and hydroxylamine the calculated velocity is a maximum at about pH 8.5. The pH behavior should therefore be distinctly different from reactions with the salt. The velocity was found to increase with higher pH up to 7.5, which is, as already mentioned, the highest pH at which the enzymatic velocity could be evaluated.

From the reaction mechanism postulated above, it was possible to conclude that the esterase would catalyze hydroxamic acid formation with esters and at a rate far exceeding that of the reaction with corresponding salts. The very different pH behavior with esters and salts is also explained. It was also expected that esters would react enzymatically with choline to produce choline esters. On the basis of this mechanism it would be anticipated that acetylcholinesterase is capable of catalyzing oxygen exchange between water and carboxylic acids. This possibility will be investigated experimentally.

In the hydrolysis of choline esters catalyzed by torpedo acetylcholin-

esterase, the acetate is hydrolyzed about 3 times as fast as the propionate and about 140 times as fast as the butyrate. These relative velocities are maintained in the reactions of the acids and esters with hydroxylamine and choline, indicating that the rate-controlling steps are the same or similar in all these reactions.

SUMMARY

Several acylation reactions catalyzed by purified acetylcholinesterase have been investigated. The picture of the active enzyme surface recently developed has been used for an analysis of the underlying mechanism.

1. Formation of hydroxamic acid from ethyl acetate is catalyzed by the enzyme some 100 times as fast as from acetic acid.

2. The rates are in both cases approximately proportional to the concentration of the reactants.

3. Hydroxamic acid formation from esters, containing 3- and 4-carbon atoms in the acyl chain, is catalyzed at a much lower rate than those containing 2-carbon atoms. The ratio of the rates is approximately the same as that observed for choline ester hydrolysis.

4. Whereas there is an optimum at pH 6.5 for hydroxamic acid formation from carboxylic acids, the reaction velocity with esters increases with pH.

5. The experimentally found pH optimum for carboxylic acid agrees with that calculated on the basis that the undissociated species is the actual reactant. This is consistent with the assumption that the carbon of the carbonyl must be markedly electrophilic to react with the basic group in the active enzyme surface.

6. In the presence of choline, the enzyme is capable of transferring the acyl group from the ester to the alcohol. Hydrolysis proceeds simultaneously.

7. In the discussion of the mechanism it is assumed that an intermediary is formed which may react either with water (or its ions) or with hydroxylamine or choline. The substance reacting with this intermediary must be nucleophilic.

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LIGHT ACTIVATION OF THE PLANT ENZYME WHICH OXIDIZES GLYCOLIC ACID*

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A wide variety of plants contains enzymes capable of rapidly oxidizing *l*-lactic, glycolic, and glyoxylic acids (1). Although the enzyme oxidizing glycolic acid was found in the green parts of many plants, it was absent from roots and etiolated seedlings. However, the active enzyme could be obtained from etiolated barley or bean seedlings that had been illuminated for several hours. This suggested that light and perhaps chlorophyll play important rôles in activating the enzyme.

When 1 mole of glycolic acid is oxidized by a highly purified lyophilized enzyme preparation from the leaves of tobacco, it yields 1 mole of formic acid, 1 mole of carbon dioxide, and takes up 1 mole of oxygen (2). The oxidation of glycolic acid by crude plant sap from tobacco leaves is accompanied by the uptake of considerably more than 1 mole of oxygen per mole of acid, but little carbon dioxide and formic acid are produced. The compounds formed during the oxidation of glycolic acid by crude enzyme preparations have been investigated.

EXPERIMENTAL

A few tests have been made with etiolated bean and corn plants grown in jars of sand. However, most experiments have been made with barley, which quickly yields a large amount of etiolated leaf tissue and has been used for investigations of chlorophyll formation (3, 4). Green barley leaves contain a very active glycolic acid-oxidizing system; since the system is unstable, it is necessary to prepare it rapidly at the designated temperature and pH. Etiolated barley plants were grown in a ventilated and totally darkened room in flats of sand supplied with Hoagland and Arnon's nutrient solution (5). Wooden flats were planted with 60 gm. of seed, and, after 7 days in total darkness at 20–25°, they yielded 100 to 110 gm. of fresh tops. After etiolation, the leaves were exposed to 450 foot candles of light from two 20 watt white fluorescent tubes. For samples at zero time, the plants were harvested quickly in very dim light by cutting off the tops about 1 inch above the sand. Three samples were

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

taken: 35 gm. for analysis of total nitrogen and measurement of enzymatic activity, 35 gm. for determination of chlorophyll, and the remainder for dry weight. In the studies of chlorophyll decolorization the barley plants were illuminated for 24 to 48 hours after the 7 day period in the dark.

In preparation for the measurement of enzymatic activity the leaves were ground with white sand in a previously chilled glass mortar; the ground material was squeezed in a double layer of gauze, and the expressed sap was caught in a cold, graduated cylinder. 7 to 8 ml. of the original sap at pH 5.6 to 5.8 were removed, centrifuged, and the ascorbic acid oxidase activity of the supernatant of this fraction was measured concurrently with the other enzymes. The rest of the sap was adjusted to pH 7.5 and centrifuged. Manometric measurements were made at pH 6.0 with ascorbic acid, pH 7.7 with glyoxylic acid and *dl*-lactic acid, and pH 8.0 with glycolic acid. Autoxidation of ascorbic acid was negligible when glass-distilled water was used; boiled sap was employed as a control upon this autoxidation. Excess sap was used for analysis of total nitrogen, and activities are expressed as Q_{O_2} (N).

During the chilling and centrifuging of the sap for the manometric measurements, the sample for chlorophyll analysis was ground with sand and extracted with acetone and ether according to the procedure of Smith (3); the extract was analyzed later for chlorophyll with a Beckman model DU spectrophotometer (6).

Results

Light Activation

Enzymatic preparations from etiolated barley, beans, and corn were tested for their ability to oxidize glycolic acid; they were inactive. The active enzyme can be demonstrated readily in leaves of normal green barley, bean, and corn plants. If the intact etiolated plants are exposed to light, the enzyme for the oxidation of glycolic acid is formed rapidly. Light has no influence on the rate or amount of oxygen uptake or on the R. Q. of cell-free enzyme preparations.

Potato—The enzyme system oxidizing glycolic acid is present in potato leaves and stems, but it is not present in the normal potato tuber. After tubers are greened by placing them in the light, they contain the active enzyme system.

Albino Corn—The absence or dearth of chlorophyll in albino corn is accompanied by an almost complete absence of the enzyme which oxidizes glycolic acid. Albino and green corn seedlings were grown in the light, and their enzymatic activity on glycolic acid was compared (Fig. 1). 1 ml. of sap from the green seedlings supported 35 μ l. of oxygen uptake, whereas 1 ml. of sap from the albino seedlings took up only 3 μ l. of oxygen

in 10 minutes. Etiolated corn does not contain the glycolic acid-oxidizing enzyme.

Rate of Activation—The effect of the period of illumination of etiolated barley upon the activation of the enzymatic system oxidizing glycolic acid is shown in Fig. 2. The enzyme is activated very rapidly during the first 6 hours of illumination, and its activity continues to increase for at least

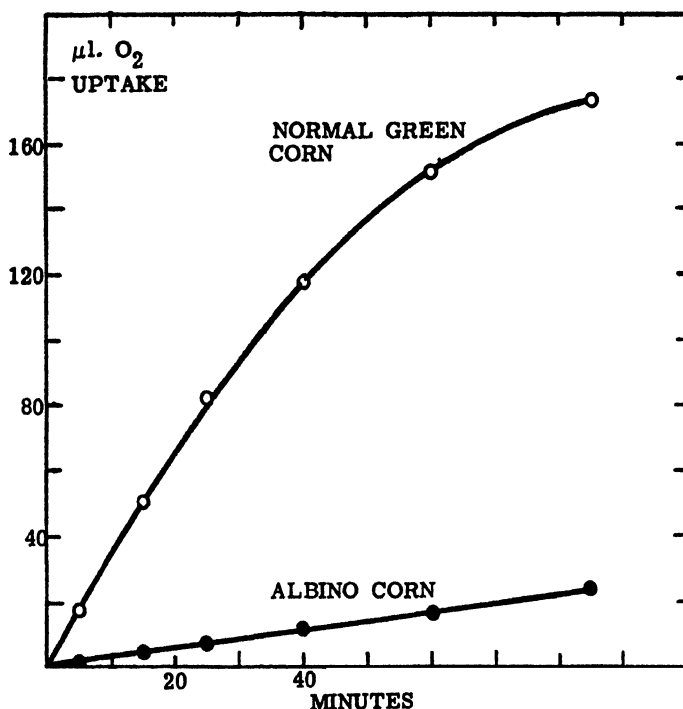


FIG. 1. Oxidation of glycolic acid by sap from green and albino corn seedlings. The flasks contained 1.0 ml. of sap from corn seedlings about 10 days old, 0.5 ml. of 0.02 M glycolic acid, 1.0 ml. of 0.1 M phosphate buffer, and 0.5 ml. of water. The pH was adjusted to 8.2. Values are corrected for respiration without added substrate.

48 hours. In contrast, the enzyme oxidizing glyoxylic acid is fully active in the etiolated barley. This difference in light activation constituted the first evidence that the enzymes oxidizing glycolic and glyoxylic acids are distinct. The enzyme which catalyzes the oxidation of *L*-lactic acid was also present in the etiolated plant. Although it was distinctly more active after the barley had been in the light for 48 hours, it was not rapidly activated initially by the light as was the enzyme which oxidizes glycolic acid. During the 48 hour period of illumination there was no change in

the activity of ascorbic acid oxidase, and there was no apparent relationship between its activity and that of the other enzymes measured.

Formation of Chlorophyll—Upon illumination of etiolated barley, the activation of the enzyme which oxidizes glycolic acid does not parallel the

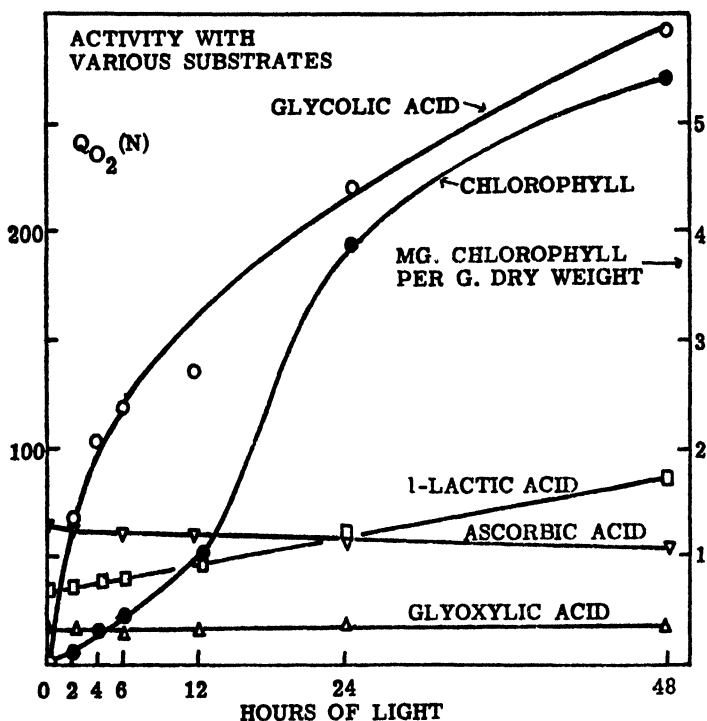


FIG. 2. Effect of light on formation of chlorophyll and on the enzymes in barley which catalyze the oxidation of glycolic, glyoxylic, *l*-lactic, and ascorbic acids. 7 day-old etiolated barley was exposed to 450 foot candles of light over a period of 48 hours. Respirometer flasks contained 1.0 ml. of sap, 0.5 ml. of 0.02 M glycolic acid or 0.04 M solution of the other substrates, 1.0 ml. of 0.1 M phosphate buffer, and 0.5 ml. of water. The rates of oxygen uptake were taken for the interval 5 to 15 minutes after addition of the substrates and were corrected for respiration without added substrate. Total nitrogen and chlorophyll analyses were run on separate aliquots.

formation of chlorophyll. During the first 6 hours, when activation of the enzyme is most rapid, the formation of chlorophyll is relatively slow (Fig. 2). The curve for the formation of chlorophyll from protochlorophyll upon illumination is very similar to that observed by Smith (4).

Activation "Spectrum"—Exploratory experiments were performed to determine whether any specific band of light activated the enzyme which oxidizes glycolic acid. Light from a 300 watt Mazda lamp in a 2 × 2 inch

slide projector was passed through filters from an Evelyn colorimeter and reflected onto etiolated barley plants. When a 420, 515, or 660 $m\mu$ filter was used, the enzyme was very active after 6 hours illumination.

Effect of Substrate on Activation of Enzyme—Benson and Calvin (7) have shown that, when algae or barley are allowed to photosynthesize $C^{14}O_2$, glycolic acid containing C^{14} is formed within 30 seconds. It is possible that the light activation *in vivo* of the enzyme might be attributed to glycolic acid production and adaptive formation of the enzyme in response to the presence of its substrate. Thus far we have been unable to check this hypothesis critically because of the low permeability of the cell membrane to glycolic acid. When glycolic acid labeled with C^{14} is infiltrated into barley tops under a vacuum at pH 6.0, it is not utilized. Myers (8) found that glycolic acid supported respiration of *Chlorella pyrenoidosa* at pH 3.8 but not at 6.8; he attributed this to enhanced permeability of the cells to the undissociated acid at the lower pH.

Respiratory Quotient and Products of Oxidation

Oxidation of Glycolic Acid by Plant Sap—During glycolic acid oxidation by plant sap or salt-precipitated enzyme there is a rapid uptake of 2 atoms of oxygen per molecule of the acid; this is often followed by continued uptake of oxygen at a decreased rate, but at a rate still much greater than that of respiration without added substrate (Fig. 3). During this oxidation little CO_2 is released. It has been shown that a molecule of glycolic acid is first oxidized to glyoxylic acid with an uptake of 1 atom of oxygen and no release of CO_2 (2). Plant sap further oxidizes the glyoxylic acid and another atom of oxygen is taken up per molecule of substrate, but little CO_2 is released and the end-product is undetermined. Tobacco, barley, bean, and tomato sap will often catalyze additional uptake of oxygen. Over a 2 hour period, barley sap has taken up as much as 3.5 atoms of oxygen (corrected for respiration without added substrate) per molecule of glycolic acid furnished. After 2 atoms of oxygen per molecule of substrate have been utilized, the uptake proceeds at a much slower rate. Sap from younger plants catalyzes more uptake of oxygen per unit of substrate than does that from older plants (Table I).

The complete oxidation of a molecule of glycolic acid to CO_2 and water requires 3 atoms of oxygen and gives an R. Q. of 1.33; thus this reaction has a much higher R. Q. than is observed experimentally in the oxidation of glycolic acid by the plant saps. The intermediate produced from glyoxylic acid after 2 atoms of oxygen have been taken up apparently is not oxalic acid. The oxidation of glycolic acid to formic acid and CO_2 would explain the 2 atoms of oxygen taken up, but the observed R. Q. of 0.1 to 0.3 does not correspond to the required value of 1.0. When C^{14} -

labeled glycolic acid was oxidized and formic acid and CO_2 were determined as described previously (2), it was found that approximately equimolar

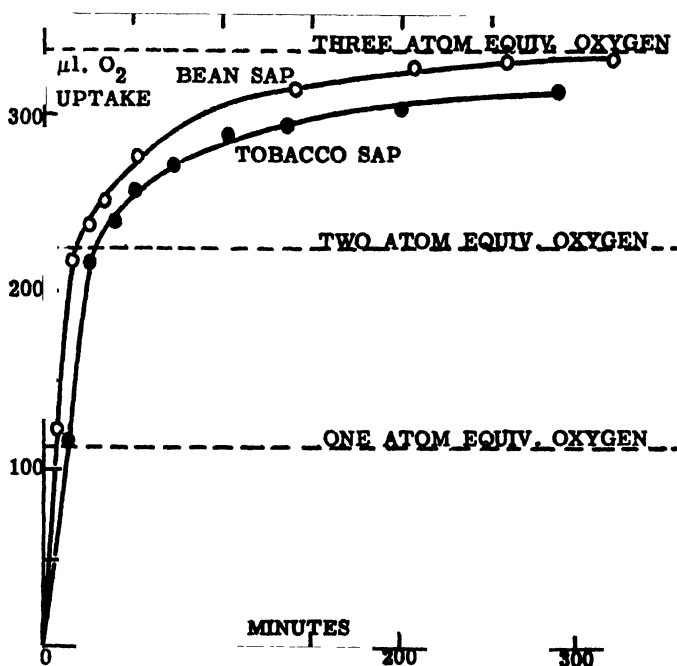


FIG. 3. Total oxygen uptake for oxidation of glycolic acid. Flasks contained 1.0 ml. of plant sap, 0.5 ml. of 0.02 M glycolic acid, 1.0 ml. of 0.1 M phosphate buffer, and 0.5 ml. of water. The pH was adjusted to 8.2. Values are corrected for respiration without added substrate.

TABLE I

Total Uptake of Oxygen during Oxidation of Glycolic Acid by Leaf Sap from Bean Plants of Various Ages

Age of bean plants, days from planting	Oxygen atoms taken up per molecule glycolic acid oxidized
19	2.96
22	2.34
24	2.17
26	2.13

In these tests the R. Q. values were less than 0.25. All the flasks contained glycolic acid at a final concentration of 0.0033 M. Oxygen uptake was corrected for respiration without added substrate.

amounts of formic acid and CO_2 were produced, but in small amounts compared to the theoretical yield. Formic acid added to the system is

not oxidized, and added $C^{14}O_2$ is not fixed in sufficient quantities to explain the low R. Q. observed. Apparently a reaction other than the oxidation of glyoxylic acid to formic acid and CO_2 observed with the purified enzyme (2) is competing for the glyoxylic acid formed from glycolic acid.

Hydrogen peroxide is not produced during the oxidation of glycolic acid, for the addition of catalase to decompose H_2O_2 , the addition of cyanide to poison any catalase already present, or the addition of ethanol to remove H_2O_2 does not alter the uptake of oxygen or the release of CO_2 .

In formulating a scheme for the oxidation of glycolic acid, $CH_2OH-COOH$, the consideration that 2 atoms of oxygen must be added to the glycolic acid to form the second intermediate restricts the possible products of the reaction to a relatively small number of compounds. It has been shown that oxalic acid, formic acid and CO_2 , and hydrogen peroxide are not the main products of the oxidation by crude enzyme preparations.

TABLE II
Effect of Substrate Concentration on Respiratory Quotient

Glycolic acid oxidized	R. Q.
<i>m.eq.</i>	
0.01	0.15
0.05	0.33
0.10	0.52
0.20	0.71

Respirometer flasks contained 1 ml. of salt-precipitated enzyme, 1 ml. of 0.1 M phosphate buffer at pH 8.1, and the designated amount of glycolic acid; atmosphere O_2 . The measurements of final CO_2 were made 30 minutes after maximum uptake of oxygen had been reached.

In light of the manometric data, an organic peroxide is a feasible product of the reaction.

When the concentration of glycolic acid supplied is increased, the R. Q. increases correspondingly (Table II). The increased output of CO_2 may arise from enhanced breakdown of a labile intermediate at higher concentrations. The portion of the labile intermediate which does not decompose may polymerize or react with protein. The observed R. Q. is not changed by varying the partial pressure of oxygen in the atmosphere of the Warburg flask, the time elapsed after exhaustion of the substrate, or the amount of a particular enzyme preparation used. The R. Q. does vary among different enzyme preparations, and it depends upon the substrate concentration.

Loss of Chlorophyll during Oxidation of Glycolic Acid—To explain how 1 molecule of glycolic acid is oxidized with the uptake of over 2 atoms of oxygen and with the release of little CO_2 it is necessary to postulate an

oxidation-reduction shuttle system and to implicate some hydrogen donor in addition to glycolic acid and its intermediates. The other substance being simultaneously oxidized, together with the glycolic acid, is apparently chlorophyll. This oxidation of chlorophyll was observed best with barley sap from plants that had grown from seed in the dark for 7 days and then in the light for 24 to 48 hours. The sap is decolorized to a pale yellow or light green color 20 to 60 minutes after the glycolic acid is added.

The system catalyzing oxygen uptake in excess of 2 atoms per molecule of glycolic acid oxidized is labile and often is not active, even in chlorophyll-containing enzyme preparations. It always is associated with the decolorization of the chlorophyll. Chlorophyll decolorized during the oxidation of glycolic acid cannot be reduced again to a green color with sodium bisulfite or ferrous chloride.

Chlorophyll has been removed from enzyme preparations from tobacco sap by extracting with ether or benzene or by repeated precipitation of the enzyme with ammonium sulfate. Such enzyme preparations catalyze about 2 atoms of oxygen uptake, whereas the green sap takes up over 2 atoms of oxygen per molecule of glycolic acid oxidized. Addition of isolated chlorophyll in a dilute alcoholic solution to an enzyme preparation, which was catalyzing the uptake of only 2 atoms of oxygen per molecule of glycolic acid, did not increase the total uptake of oxygen. Apparently, to be effective, the chlorophyll must be in its native physical state. Although the data suggest that the system may have been inactivated by the removal of chlorophyll *per se*, these experiments do not eliminate the possibility that treatments which removed chlorophyll directly inactivated the enzymes responsible for the uptake of oxygen in excess of 2 atoms per molecule of the substrate.

Effect of Cyanide—The uptake of oxygen beyond 2 atoms per molecule of glycolic acid is inhibited by high concentrations of cyanide (Fig. 4). Although at concentrations lower than 0.001 M cyanide does not affect appreciably the total oxygen uptake or the R. Q., at 0.01 M concentration it increases the initial rate of glycolic acid oxidation by as much as 60 per cent and the rate of glyoxylic acid oxidation by the salt-precipitated enzyme about 90 per cent. Cyanide at 0.01 M concentration completely inhibits the uptake of the 3rd atom of oxygen; in addition, the total oxygen uptake falls somewhat short of 2 atoms per molecule of glycolic acid added. Cyanide also inhibits the decolorization of chlorophyll. In the presence of cyanide the R. Q. remains small.

Isolation of Decolorized Chlorophyll—Chlorophyll, partially decolorized in barley sap-oxidizing glycolic acid, was extracted with ether, and the ether was removed under a vacuum in the presence of aqueous methanol. The aqueous methanol solution was extracted with a mixture of 10 parts

Skellysolve B and 1 part benzene and the methanol was removed by washing three times with water. The Skellysolve B and benzene solution was dried over anhydrous sodium sulfate and chromatographed on a powdered sucrose column. A large yellow band moved down the column just ahead of chlorophyll *a*. Under ultraviolet light the "yellow chlorophyll" section of the column was scarlet-red and the sections containing chlorophylls *a* and *b* gave a characteristic deep red fluorescence. The

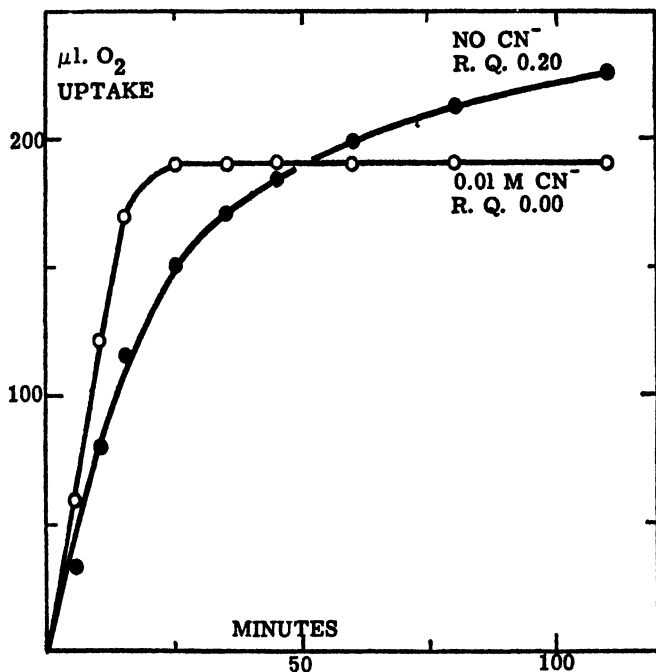


FIG. 4. Effect of cyanide on the oxidation of glycolic acid by sap from tobacco leaves. Flasks contained 1.0 ml. of sap, 0.5 ml. of 0.02 M glycolic acid, 1.0 ml. of 0.1 M phosphite buffer, and 0.5 ml. of 0.06 M NaCN or 0.5 ml. of water. The pH was adjusted to 8.4.

section of the column containing the "yellow chlorophyll" was extracted with a mixture of 10 parts of ether and 1 part of methanol, and the entire chromatographic procedure was repeated with this extract. On the second chromatogram the yellow chlorophyll was completely resolved into two components. The absorption spectrum of each component in ether was determined with a Beckman model DU spectrophotometer; the spectra were not appreciably different. It is likely that these two yellow chlorophylls are the oxidation products of chlorophylls *a* and *b*. Fig. 5 gives the spectrum for the faster moving yellow chlorophyll. The principal

absorption maximum is near $440\text{ m}\mu$ and a smaller peak appears at $665\text{ m}\mu$. In the yellow chlorophyll, the small peak at $665\text{ m}\mu$ replaces the usual strong absorption of chlorophyll in the red, and the characteristic protochlorophyll peak (3) at $625\text{ m}\mu$ is absent. Rabinowitch and Weiss (9) have reported that ferric chloride oxidizes chlorophyll to a yellow product. If it has not been exposed to light or dissolved in a solvent containing as much as 20 per cent water, it may be reduced again to green chlorophyll by the addition of ferrous chloride. Their yellow chlorophyll

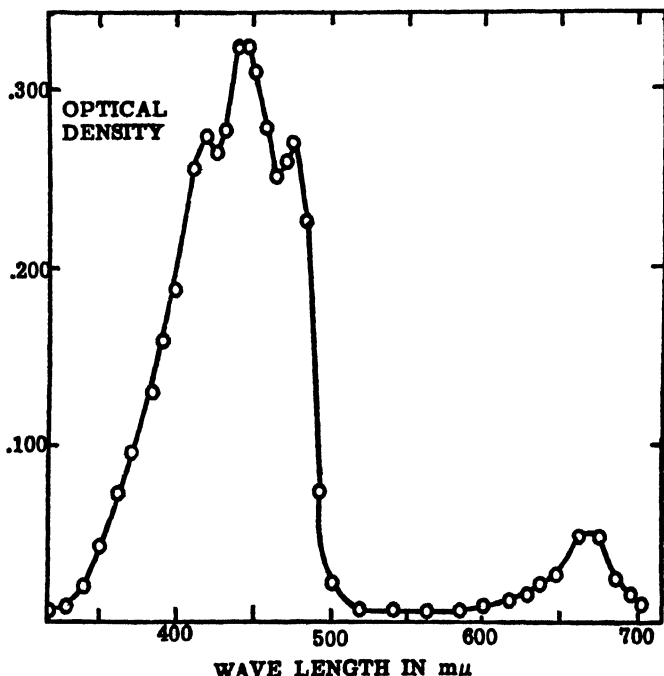


FIG. 5. Absorption spectrum of the yellow chlorophyll formed during the oxidation of glycolic acid. The yellow chlorophyll which moved most rapidly on a powdered sucrose column was measured in ether solution with a Beckman spectrophotometer.

had approximately the same absorption spectrum as the yellow chlorophyll obtained during oxidation of glycolic acid. The yellow chlorophyll formed during oxidation of glycolic acid is not converted to green chlorophyll by ferrous chloride; this was to be expected (9) as it was formed in an aqueous solution and handled in the light.

DISCUSSION

The enzyme oxidizing glycolic acid to glyoxylic acid is present in the green parts of plants, but is absent from roots and from etiolated leaves.

It is formed or activated upon exposure of etiolated leaves to light. This is perhaps the first report of the complete activation of an enzyme by light, although stimulation of enzymatic activity by light has been reported earlier (10-12). The activation of the enzyme is most rapid during the first 6 hours of illumination of etiolated barley. The enzyme cannot be activated by illumination of the sap from etiolated plants. Very little light is necessary to activate the enzyme to a measurable degree, for it was necessary to have a room completely dark before etiolated tissue devoid of the enzyme could be obtained. Once formed, the enzyme which oxidizes glycolic acid is not destroyed by putting a green plant in the dark until it dies.

The mechanism of the light activation of the enzyme is not known. It is possible that light actually may cause a spatial rearrangement of the protein, a photoreduction at some point, the removal of a blocking agent, or the formation of a cofactor. The possibility of substrate activation of the enzyme has not been determined because glycolate does not readily cross the cell wall. Chlorophyll formation does not coincide with light activation of the enzyme. Active enzyme preparations can be freed of their chlorophyll without losing their ability to oxidize glycolic acid. Bernheim and Dixon (11) have attributed the light stimulation of xanthine oxidase to the formation of H_2O_2 .

The fact that sap from etiolated plants catalyzes the oxidation of glyoxylic and *l*-lactic acids but not glycolic acid indicates that more than one enzyme may be responsible for the oxidation of these three substrates, contrary to our previous report (2). Glyoxylic and lactic acids are similar structurally; both are α -hydroxy acids, for glyoxylic acid actually exists as the monohydrate $CH(OH)_2COOH$. It is conceivable that one enzyme might oxidize both acids, but the constancy of activity on glyoxylic acid and the increase of activity on *l*-lactic acid with time of illumination of etiolated barley substantiate the idea that two enzymes are concerned in addition to the one oxidizing glycolic acid.

It is interesting that such a limited amount of enzyme for the oxidation of glycolic acid is present in albino corn, for this plant tissue has been exposed to light and normally should contain the enzyme. In albino corn, there is little or no chlorophyll or protochlorophyll, no photosynthetic mechanism, little catalase activity (13), and little or no enzyme that oxidizes glycolic acid. The concurrent absence of these functions suggests that the glycolic acid-oxidizing system is associated with photosynthesis. The etiolated plant or the albino plant is capable of living until the reserves from the seed or external nutrients are exhausted. Therefore, the enzyme that oxidizes glycolic acid is not essential for the growth or respiration of the plant. Although present information does not permit us to assign a definite function to the enzyme in the economy of the plant, it

may function as an alternate respiratory system which is not entirely essential to the plant or it may be concerned with some phase of photosynthesis.

The following points are salient to the thesis that glycolic acid and its oxidation system are involved in the over-all process of photosynthesis: (a) the enzyme which oxidizes glycolic acid is present only in green parts of plants, (b) the enzyme is activated *in vivo* by light, (c) during glycolic acid oxidation *in vitro* chlorophyll is decolorized, apparently being oxidized by the end-product of the oxidation of glycolic acid, (d) Benson and Calvin (7) have shown that glycolic acid is one of the earliest products to contain C^{14} after a short exposure of plants to $C^{14}O_2$.

The products of the oxidation of glycolic acid by tobacco sap or by crude salt-precipitated enzyme remain undetermined. The first reaction, as with the lyophilized enzyme, is the oxidation of glycolic acid to glyoxylic acid, but little CO_2 and a correspondingly small amount of formic acid are produced as end-products. However, the uptake of oxygen is still at least 2 atoms per molecule of glycolic acid oxidized. Apparently a reaction other than the oxidation of glyoxylic acid to formic acid and CO_2 is competing for the glyoxylic acid formed; such competition is absent in the lyophilized preparation. All of the compounds that seem logical products from glycolic and glyoxylic acid oxidation have been investigated. Carbon dioxide and formic acid are found in small quantities, but oxalic acid and hydrogen peroxide have not been detected. The possibility that a peroxide of glyoxylic acid is formed has not been eliminated.

The oxygen uptake over 2 atoms per molecule of glycolic acid oxidized and the decolorization of the chlorophyll are inhibited by 0.01 M cyanide. The products of the oxidation of chlorophyll are two yellow chlorophylls which resemble the yellow chlorophylls obtained by oxidizing chlorophyll with ferric chloride.

The amount of chlorophyll on a molar basis in the manometric experiments was small in relation to the concentration of the glycolic acid added. Therefore, when the uptake of oxygen during oxidation of glycolic acid approached 3 or more atoms, the 3rd atom of oxygen was far in excess of that needed to oxidize the chlorophyll. The chlorophyll itself may have been reduced by an independent hydrogen donor, for it was not completely decolorized except in preparations from etiolated barley that had been greened for a short time.

Kolesnikov (14) has reported observations similar to ours regarding glycolic acid oxidation by barley sap. He found that barley sap oxidizes glycolic acid, and, though he did not isolate glyoxylic acid or derivatives of it, he proposed that it was the product of the oxidation. Kolesnikov

(15) also observed that barley sap, supplied with glycolic acid as a substrate, supported an oxygen uptake far in excess of that required to oxidize glycolic acid to glyoxylic acid or even to CO_2 . During oxidation of glycolic acid he found that chlorophyll was destroyed and suggested that because of the accumulation of organic peroxides in a suspension of barley leaves an active organic peroxide was responsible for the oxidation of the chlorophyll.

SUMMARY

1. The enzyme catalyzing the oxidation of glycolic acid to glyoxylic acid is activated by light in intact etiolated plants. The enzyme is not present in etiolated barley, beans, or corn, and in very limited concentration in albino corn. It is present in these plants in their normal green counterparts. It is absent from roots and from potato tubers, but is present in illuminated tubers which have become green. The activation of the enzyme in etiolated barley is most rapid during the first 6 hours of exposure to light, and the activation does not follow chlorophyll formation. The enzyme is activated by white, red, blue, or green light. It does not disappear from green plants placed in the dark. Light has no effect upon glycolic acid oxidation by sap from the green plant.

2. The enzymes catalyzing the oxidation of ascorbic acid, glyoxylic acid, and *l*-lactic acid are present in etiolated plants; the activity of the first two enzymes is unaffected, and of the third is somewhat increased by exposure to light.

3. Several lines of evidence suggest that glycolic acid and the enzymatic system for its oxidation may be concerned with photosynthesis.

4. Tobacco sap or crude enzyme preparations use 2 or more atoms of oxygen per molecule of glycolic acid oxidized. Little CO_2 and a correspondingly small amount of formic acid are produced. Glyoxylic acid is the first intermediate product of the oxidation. The respiratory quotient increases with increasing substrate concentration. Investigation of the possible end-products and examination of the respiratory data indicate that glyoxylic acid possibly is further oxidized to a peroxide.

5. Concurrent with the oxidation of glycolic acid by plant sap or crude enzymes, there is a decolorization of chlorophyll and an uptake of oxygen in addition to the 2 atoms of oxygen which are rapidly utilized in the oxidation of a molecule of glycolic acid. The uptake of extra oxygen is more pronounced when sap from young plants is used; it is inhibited by 0.01 M cyanide or by removal of the chlorophyll. Chlorophyll is oxidized to two yellow chlorophylls which may arise from chlorophylls *a* and *b*. The yellow chlorophylls have characteristic absorption spectra which differ from that of protochlorophyll.

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NET PRODUCTION OF SERUM ALBUMIN BY LIVER SLICES

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Direct observation of protein synthesis *in vitro* is made difficult by the fact that the total protein content of tissue slices decreases upon incubation, owing to the predominance of proteolytic reactions. This has necessitated the use of isotopes to demonstrate that protein formation or "turn-over" is taking place under such conditions.

Experiments with radioactive carbon, reported in a previous paper (1), demonstrated the incorporation of amino acids into serum albumin by chicken liver slices. This incorporation was found to proceed at a rapid rate, which suggested that the formation of this specific protein might be demonstrated in these liver slice systems without the use of isotopes.

An immunological method was developed by which the small amounts of serum albumin present in liver slices could be determined. The total amount of serum albumin was found to increase in each of twenty experiments, at an average rate which corresponded roughly to observed rates of albumin production *in vivo*. Studies were made of the effects on this rate of production of changes in the ionic environment and of addition to the incubation medium of substrates, inhibitors, and hormones.

EXPERIMENTAL

Methods

Several investigators have reported the application of quantitative immunological techniques to the determination of serum albumin in serum, urine, and edematous and cerebrospinal fluids (2-4). These methods are extremely sensitive and give results which have been found to agree well with electrophoretic determinations.

Preparation of Standard Albumin—Albumin was isolated from chicken

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plasma by a procedure which included two series of fractionations with ammonium sulfate, and three fractionations with alcohol under various conditions. Electrophoretic analysis of the final product at pH 8.0 showed only one peak, which was estimated to contain not more than 0.9 per cent of impurity. A test for immunological homogeneity was performed according to the antibody gel method of Oudin (5), in which bands of precipitate representing each antigenic component in the solution tested are observed to diffuse into gelled antiserum. Although such a procedure shows only a minimal number of components with certainty, the albumin fraction appeared to be homogeneous by this test.

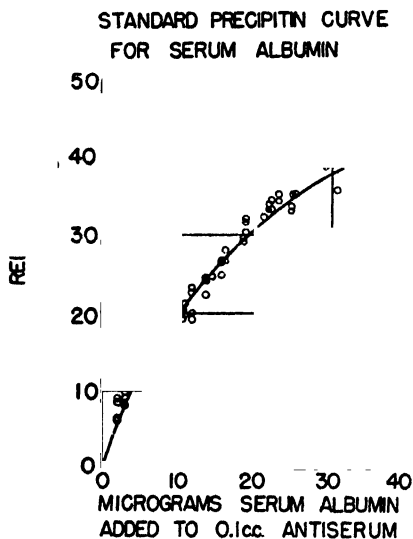


FIG. 1. The relation between the amount of standard albumin added to a fixed volume of antiserum and the amount of precipitate resulting.

Rabbit Antiserum—Portions of this albumin were injected into five rabbits three times weekly for 4 weeks. The weekly doses were 1.5, 2.2, 2.6, and 3.6 mg. 5 days after the last injection, blood was drawn, under sterile conditions, from all of the rabbits and the sera were pooled. This combined antiserum gave a positive ring test with the standard albumin diluted 1:800,000 and contained approximately 0.5 mg. of antibody nitrogen per cc.

Microdetermination of Serum Albumin—The relation between the amount of standard albumin added to a fixed volume of antiserum and the amount of precipitate resulting is plotted in Fig. 1. In order to conserve antiserum, a microprocedure was used for determination of the amount of precipitate. This consisted of a modification of the method described by

Johnson (6), in which the amount of dichromate reduced in the presence of 66 per cent H_2SO_4 at 100° is taken as a measure of the amount of organic material present. The results are consistent when only one type of substance, such as protein, is analyzed.

For determination of serum albumin, slices were homogenized for 2 minutes at 0° with 4 volumes of saline, or with the medium with which they had been incubated, in a Potter-Elvehjem glass homogenizer. The slice homogenates, or in some cases the isolated incubation media, were centrifuged at 5° at $10,000 \times g$ for 10 minutes. The layer of lipide which rose to the top was removed, and the centrifugation repeated.

Two aliquots of the clear supernatant were added by constriction pipette to 0.083 cc. of antiserum, under the same conditions used in determining the standard curve. These aliquots were chosen to give precipitates of widely differing sizes and the results were averaged in order to minimize errors in the manner in which the standard curve was drawn. Pyrex test-tubes, 1×12 cm. with tapered bottoms, were employed. The final volume was adjusted to 0.26 cc. by the addition of 0.9 per cent NaCl.

After 24 hours the antibody precipitates were centrifuged for 5 minutes at $1100 \times g$ and washed twice with 0.11 cc. of saline. A third washing of the precipitate and the walls of the tube with water was employed in order to remove chloride, which was found to interfere in the dichromate reaction.

To each tube was added 0.187 cc. of 0.251 N $\text{K}_2\text{Cr}_2\text{O}_7$, followed by 0.47 cc. of 95 per cent H_2SO_4 . The contents were stirred occasionally with a glass rod during the 30 minute period at 100° . After cooling, 4 cc. of water were added and the solution was mixed. The density was read in a Beckman spectrophotometer at $445 \text{ m}\mu$ against a similar solution to which sodium hyposulfite had been added to reduce the dichromate completely. The densities of test solutions were subtracted from the density of an unreduced standard, and the microequivalents of dichromate reduced by the precipitate were calculated by proportion.

By this method 10 to 30 γ of serum albumin could be determined with a precision of ± 5 per cent. Analyses for albumin in samples of chicken serum gave 90 per cent of the value obtained by the salt fractionation method of Howe (7). Determinations made on saline liver extracts to which known amounts of albumin had been added gave the expected analytical results.

Incubation Procedure—Immediately after killing the animal, chicken livers were cut into 0.5 mm. slices with a Stadie slicer and incubated in Erlenmeyer flasks containing medium with the following composition: Na 135, K 10, Ca 10, Cl 125, HCO_3 40 mm per liter, in conjunction with a gas phase containing 5 per cent CO_2 -95 per cent O_2 .

Unwashed liver slices were found to contain about 1.5 mg. of serum albumin per gm. of wet liver. In order to lower the initial level, the slices were routinely washed for an hour by rocking in a 500 cc. flask containing the above medium. The slices were then blotted on filter paper, and portions of about 2 gm. were accurately weighed and placed in 125 cc. flasks containing 10 cc. of medium. A sample was also taken for determination of initial albumin level, which had been lowered to about 0.3 mg. per gm. by the washing procedure.

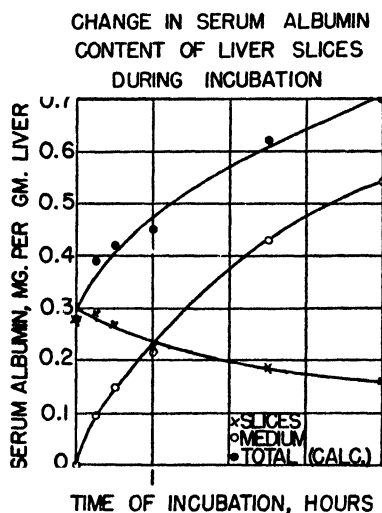


FIG. 2. The increase in the amount of serum albumin during incubation. Both the medium and slices were analyzed separately for the albumin level.

Results

In all of the experiments, increases in the amount of serum albumin present in the system were found after 4 hours of incubation. The average rate of increase in twenty experiments was 0.12 mg. per gm. of liver per hour. Only 20 per cent of the livers gave increases of less than 0.08 mg. per gm. per hour.

Fig. 2 shows the manner in which this increase occurred during a typical experiment. In this experiment both medium and slices were analyzed separately for the albumin level. Each time which is plotted represents a separate flask. Fig. 2 shows that albumin continues to be washed from the slices as the incubation proceeds, and appears in the medium at a rate which is more rapid during the 1st hour than in the latter stages of incubation.

Effect of Ions and Inhibitors—Table I summarizes the changes in the

net increase of serum albumin caused by variation in the ionic composition of the incubation medium and by addition of various metabolic inhibitors. The presence of phosphate was inhibitory, with or without the presence of bicarbonate. Either calcium or magnesium was required for maximal albumin production. While this requirement for divalent cation may simply reflect the dependence of albumin formation on general intra-

TABLE I
Effect of Ions and Inhibitors on Net Production of Serum Albumin

Condition	Production in 4 hrs. compared to control flask per cent
10 mM per liter PO_4 added.....	39
Ca replaced by Mg	94
“ reduced to 2.5 mM per liter.....	105
Divalent cation omitted.....	74
K replaced by Na.....	95
Na “ “ K.....	60
240 mOsM per liter.....	99
260 “ “ “	100
330 “ “ “	102
O_2 replaced by N_2	23
Cyanide, 0.002 M.....	0
“ 0.0002 M.....	53
Azide, 0.005 M.....	6
Arsenite, 0.001 M.....	10
“ 0.0031 “.....	27
Iodoacetate, 0.001 M.....	10
“ 0.0001 “.....	82
Dinitrophenol, 0.0005 M.....	2
“ 0.0001 “.....	54
Arsenate, 0.01 M.....	26
“ 0.031 “.....	25
Fluoride, 0.005 M.....	44
Malonate, 0.01 “.....	94

cellular enzymatic activity, it is of interest to note that magnesium has been found necessary in model systems which effect the formation of peptide bonds (8-11).

The effects caused by the different inhibitors tested show that the appearance of albumin is a process dependent upon a supply of energy generated within the cell. The inhibitions caused by the two concentrations of dinitrophenol used are similar to the effects which this compound has on the incorporation of labeled alanine into liver slice proteins (12).

Since the dinitrophenol appears to affect cellular reactions by blocking the transfer of phosphate bond energy (13), the results found here imply that high energy phosphate is involved in the formation of proteins. Other evidence for the importance of phosphate bond energy is found in the functioning of adenosinetriphosphate in the formation of peptide bonds in model systems (8-11).

It is interesting to note that high concentrations of malonate, which were without effect in the present system, also failed to inhibit the active

TABLE II
Simultaneous Appearance of Albumin and Incorporation of Radioactivity

	Condition	Initial level	Level after 4 hrs. incubation
Observed data	Amount of serum albumin present, <i>mg. per gm. liver</i>	0.28	0.70
	Total radioactivity in serum albumin, <i>c.p.m. per gm. liver*</i>	0	724
Calculated data	Specific activity of albumin carboxyl groups, <i>c.p.m. per mg. albumin†</i>		1,030
	Specific activity of albumin carboxyl groups, <i>c.p.m. per mm CO₂‡</i>		125,000
	Specific activity of dicarboxylic amino acid carboxyl groups, <i>c.p.m. per mm CO₂§</i>		375,000

* Determined by counting CO₂ released from hydrolysate by treatment with ninhydrin, based on specific activity of bicarbonate of 1,000,000 c.p.m. per mm of CO₂.

† Calculated by dividing the total radioactivity by the amount of albumin.

‡ Calculated by multiplying the figure of the previous line by 121, the average residue weight found for serum albumin.

§ Calculated by multiplying the figure of the previous line by 3.0, the ratio of specific activities found (1) for the CO₂ released by ninhydrin from the dicarboxylic amino acids to that released from the total hydrolysate.

intracellular concentration of amino acids by rat diaphragms (14), although this system was sensitive to all other inhibitors tested. This comparison suggests that the intracellular concentration of amino acids is an important factor in the synthesis of proteins.

Effect of Substrates and Hormones—No consistent effects were observed upon the addition of amino acid mixtures or oxidizable substrates to the media during incubations. The liver slice system appears to be complete in itself with regard to the supply of amino acids and of energy-yielding compounds required.

No significant change in the rate of albumin production was found upon

addition to the incubation medium of insulin, thyroxine, growth hormone, adrenal extract, cortisone, or desoxycorticosterone. This does not preclude hormonal influences in the control of the formation of albumin, since the demonstration of hormonal effects by addition *in vitro* has rarely been accomplished, possibly owing to such factors as destruction of the hormone, failure of penetration, or insufficient time for action.

Incorporation of Radioactive Carbon into Albumin—The incorporation of large amounts of labeled amino acids into serum albumin concomitant with a net increase would serve to indicate that this albumin is actually newly formed protein, and not merely preformed albumin which had been released from some complex within the cell. Accordingly, an experiment was performed in which were determined both the total amount of albumin present and the amount of incorporation of carbon from $C^{14}O_2$ into the dicarboxylic amino acids of the albumin molecule. The incubation and counting procedures have been described previously (1). The amount of radioactivity incorporated was determined by precipitating aliquots of albumin with antiserum, washing and hydrolyzing the precipitates, and counting the CO_2 released from the hydrolysate by treatment with ninhydrin.

From the measurements on the serum albumin present after 4 hours of incubation, it could be calculated that 37.5 per cent of the carboxyl groups of aspartic and glutamic acids which are released by ninhydrin contained labeled carbon (Table II). If it were assumed that the albumin present at the start of incubation underwent no turnover, this figure would be raised to 63 per cent for the dicarboxylic amino acids of the albumin produced during the incubation. This high degree of incorporation strongly suggests that the appearance of serum albumin as observed in the experiments reported here does represent formation of new protein.

DISCUSSION

A number of earlier reports have been concerned with the incorporation *in vitro* of labeled compounds into the proteins of tissues and homogenates. These studies furnished suggestive evidence for the synthesis of new protein molecules. In the present experiments, the application of immunological techniques combined with radioactivity measurements has yielded more direct proof for this synthetic process.

By using the average rate observed for albumin production by slices, a rough calculation can be made comparing the rate of production *in vitro* with the rate of albumin replacement indicated for the intact animal by isotope studies *in vivo*. The average observed liver size was 1.8 per cent of the body weight. Hence a 2.5 kilo chicken would have a liver weighing 45 gm. Based on the average rate of serum albumin production of 0.12

mg. per gm. of liver per hour, a liver of this size would form 5.4 mg. of serum albumin per hour, or 129 mg. per day.

A 2.5 kilo chicken would have a total circulating albumin content of 1730 mg., assuming a blood volume of 7 per cent, a hematocrit of 0.45, and an albumin level in serum of 1.8 per cent. Hence the liver could replace the total circulating albumin in $1730 \div 129$ or 13.4 days. This calculation gives a half life for serum albumin in the chicken in substantial agreement with the turnover rates determined for various animals by observation of the rates of incorporation of isotopes *in vivo* (15-17).

While this calculation can give only an order of magnitude, the demonstration that the liver can produce albumin at a rate comparable to the rate of albumin replacement in the intact animal suggests that the liver, unaided, can make all or a large part of the body's requirement of albumin. This concept has previously been indicated by experiments involving operative procedures (18) and isotope studies on perfused livers (19). A further implication of this calculation is that the incorporation of labeled amino acids observed *in vivo* actually represents the formation of new molecules and not merely replacement of amino acids in existing molecules, as suggested earlier by the studies of Heidelberger *et al.* on antibodies of passive immunization (20).

SUMMARY

1. An immunological technique was developed by which small amounts of serum albumin in chicken liver slices and their incubation media could be determined. The standard albumin for this procedure was obtained by isolation from chicken serum.

2. It was shown that chicken liver slices, upon incubation in bicarbonate medium, produce a net quantity of serum albumin of about 0.12 mg. per gm. of liver per hour.

3. Investigations were made of the effect on albumin production of variations in the ionic composition of the incubation medium and of addition to the medium of inhibitors, substrates, and hormones.

4. Demonstration of incorporation of large amounts of $C^{14}O_2$ into the dicarboxylic amino acids of this albumin is further evidence that the observed appearance of albumin actually represents the formation of new protein.

The authors wish to thank Mr. M. J. Budka for carrying out electrophoretic analyses on the standard albumin.

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INSULIN METHYL ESTER

II. ULTRACENTRIFUGAL OBSERVATIONS OF THE ESTERIFICATION PROCESS

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(Received for publication, May 15, 1950)

In the preceding paper of this series (5), the preparation and some chemical and biological properties of insulin methyl ester were described. The present communication records the effect of esterification on the molecular aggregation of insulin, as measured by sedimentation analyses in solutions of varying ionic strengths. Since recent investigations have shown that insulin tends to dissociate into units of approximately 12,000 (1, 3, 4), or even 6000 molecular weight (2), as the net positive charge of the protein is increased, it was expected that a similar effect should accompany the esterification of the carboxyl groups of the protein and that under such conditions the range of dissociation should be shifted toward higher pH values. This has, indeed, been verified as documented by the data of the present paper, and by others to be given in the third paper of this series.¹

EXPERIMENTAL

The methods of preparation of insulin methyl ester have already been described (5). The lyophilized preparations were dissolved directly in the desired buffer. Measurements of sedimentation rate were performed in the electrically driven ultracentrifuge, model E, constructed by the Specialized Instruments Corporation (7).

All measurements were made at 59,780 r.p.m. at temperatures varying between 20–29°. Sedimentation constants were calculated in the usual manner (6, 8) and corrected for solvent viscosity and density ($s_{20,w}$). In the present experiments, measurements of sedimentation rate were conducted on solutions containing 1 per cent protein dissolved in 0.02 M acetate buffer of pH 3.7 ± 0.1 . Sodium chloride was added to this buffer to a final ionic strength of 0.1 or 0.2, respectively.

The choice of pH 3.7 was based on *a priori* reasoning of the effect of esterification of carboxyl groups on the pH range of molecular disaggregation. In more alkaline solutions, insulin methyl ester presumably would

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¹ Manuscript in preparation.

occur largely in the polymerized form, whereas in more acidic solutions insulin itself is largely disaggregated (1, 2). The pH range chosen here was expected, therefore, to show most distinctly the change in aggregation on progressive esterification.

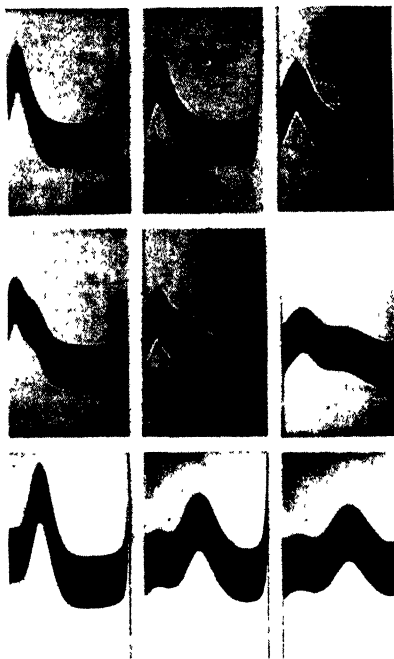


FIG. 1. Ultracentrifugal patterns of 1 per cent solutions of insulin methyl esters in acetate buffer of $\text{pH } 3.7 \pm 0.1$; centrifugal field, $254,500 \times g$. Upper row, 24 hours esterification in 0.1 N HCl-methanol at 25° . Measurements at 0.1 ionic strength after 32, 64, and 80 minutes of sedimentation, respectively. Note dominance of α component. Middle row, same protein preparation, measured at 0.2 ionic strength after 24, 48, and 64 minutes of sedimentation, respectively. Note presence of β component. Bottom row, 7 days esterification in 0.1 N HCl-methanol at 20° . Measurements at 0.1 ionic strength after 40, 56, and 72 minutes of sedimentation, respectively. Note preponderance of β component after prolonged incubation in esterifying media.

Results

Whereas crystalline insulin is essentially monodisperse in the ultracentrifuge, this is not necessarily true of its esters. Fig. 1 illustrates the types of sedimentation patterns which have been observed on insulin, esterified to varying degrees, and dissolved in solutions of 0.1 and 0.2 ionic strength, respectively. The slower moving component, herein referred to as the α component, usually predominates and shows less boundary spreading than does the faster moving β component.

The dependence of the sedimentation constants on the extent of esterification by methanol in the presence of 0.1 *N* hydrochloric acid, at 25°, is shown in Fig. 2. The measured constants ($s_{20,w}$) are plotted separately for measurements at 0.1 and 0.2 ionic strength, respectively. At 0.1 ionic strength, the sedimentation constant of the α boundary decreases with progressive esterification, reaching a value of 1.5 *S* in 1 per cent solution after 2 days of esterification. On esterification for periods much longer than those represented in Fig. 2, still lower sedimentation constants are reached, but the relative amount of the α component becomes so small that

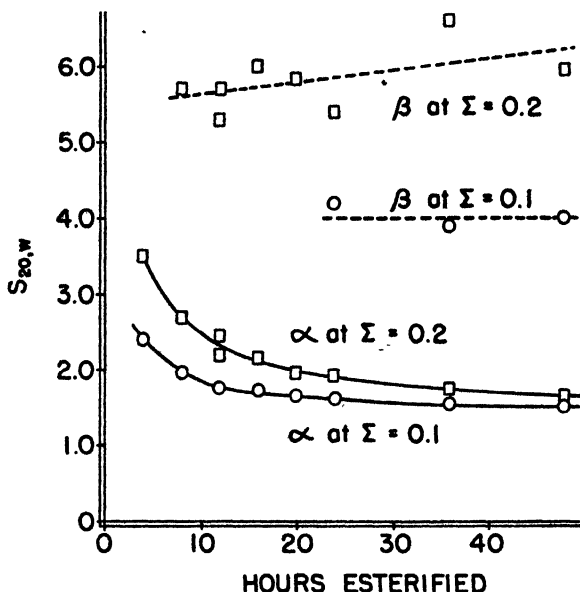


FIG. 2. Changes in the ultracentrifugal characteristics of insulin during esterification at 25° in 0.1 *N* HCl-methanol. Sedimentation constants of α and β components in solutions of pH 3.7 ± 0.1 of 0.1 (O) and 0.2 (□) ionic strength.

its sedimentation rate cannot be measured with the required precision. At 0.2 ionic strength, the sedimentation constants of the α component are higher, but, with progressing esterification, they appear to converge toward a similar limiting value.

The β component is revealed in solutions of 0.2 ionic strength after a few hours of esterification, and in solutions of 0.1 ionic strength after approximately 24 hours of esterification. Because of the relative diffuseness of the sedimenting boundary, the sedimentation constant of the β component could be measured only with limited precision. Within these limits, the sedimentation constant shows no significant dependence on the extent of esterification.

Phenomena similar to those just described are encountered if esterification is carried out at lower acid concentration (0.0125 M) or at lower temperature (0°). These results are shown in Figs. 3 and 4, respectively. In these cases, esterification and the concomitant molecular-kinetic changes occur more slowly. The observations were not continued sufficiently long to study the behavior of the β component over a wide range.

In order to express the data obtained under varying experimental conditions on a common basis of reference, the measured sedimentation constants were plotted against the methoxyl content, as shown in Fig. 5. While

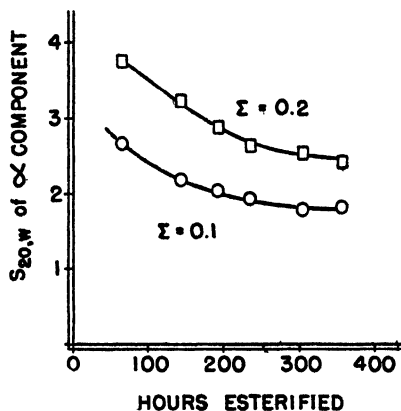


FIG. 3

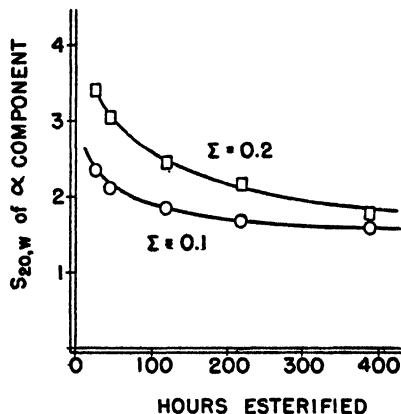


FIG. 4

FIG. 3. A plot of the sedimentation constant of the α component against the time of esterification at 25° in 0.0125 N HCl-methanol. Measurements in solutions of pH 3.7 \pm 0.1 of 0.1 (O) and 0.2 (\square) ionic strength.

FIG. 4. A plot of the sedimentation constant of the α component against the time of esterification at 0° in 0.1 N HCl-methanol. Measurements in solutions of pH 3.8 \pm 0.1 of 0.1 (O) and 0.2 (\square) ionic strength.

the scattering of the experimental points, originating from chemical rather than from physical measurements, is appreciable, there does not appear to be any systematic difference between the results obtained under the various conditions of esterification. It is apparent from these data that the sedimentation rate of the α component is a function of the degree of esterification and decreases as the latter increases.

Although the sedimentation constant of the β component appears to be unrelated to the methoxyl content of the ester, the relative amount of this component increases with progressive esterification. This is shown in Fig. 6 in which, in solutions of high and low ionic strength, the per cent of the β component in the total system is compared to the value of its sedimentation constant obtained at 0.1 ionic strength. The latter quantity was used as a basis of reference, since it was determined with higher precision

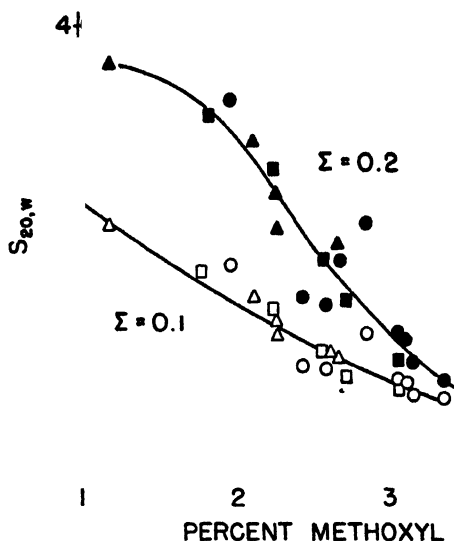


FIG. 5. Sedimentation constants of the α component of esterified insulin, measured at $\text{pH } 3.7 \pm 0.1$, plotted as a function of the methoxyl content. Open symbols, 0.1 ionic strength; filled symbols, 0.2 ionic strength. The conditions of esterification were as follows: (O, ●), 0.1 N HCl-methanol, 25°; (Δ , \blacktriangle), 0.0125 N HCl-methanol, 25°; (\square , \blacksquare), 0.1 N HCl-methanol, 0°.

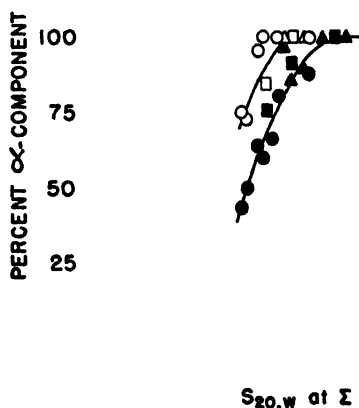


FIG. 6. Relative concentrations of α and β components, expressed as percent abundance of α , in preparations of insulin methyl ester of varying degrees of esterification. These preparations are characterized by the sedimentation constants of the α component (plotted along the abscissa) at 0.1 ionic strength, $\text{pH } 3.7$. The meaning of the symbols is the same as in Fig. 5.

than was the methoxyl content. The points denoted by various symbols, corresponding to various conditions of esterification, show that the occurrence of the β component is independent of the temperature of esterifi-

cation and of acid concentration, and, hence, that its appearance cannot be suppressed by variations of these external factors.

DISCUSSION

The present ultracentrifugal measurements indicate clearly that esterification of the carboxyl groups of insulin leads to a progressive decrease in the sedimentation rate of the α component, gradually approaching a limiting value of about 1.5 S in 1 per cent protein solution. This phenomenon is analogous to that seen when the net positive charge of native insulin is increased by a decrease in pH, and is to be ascribed to the same effect, *i.e.*, a progressive dissociation of the insulin polymer into its monomeric units (1-4). Since in preparations of incompletely esterified insulin the α component is never resolved into separate boundaries corresponding to monomers and higher aggregates, the mutual reversion of monomers and polymers must be fast with respect to the disturbance of this equilibrium by sedimentation in the ultracentrifugal field. Comparison of measurements in solutions of 0.1 and 0.2 ionic strength, respectively, indicates a shift of the equilibrium toward the aggregated form with increasing electrolyte concentration. These findings are entirely analogous to those observed for the dissociation of native insulin at higher acidity.

The β component appears to represent a secondary aggregation product. Although its formation is also promoted by increasing electrolyte concentrations, it does not seem to partake in a rapidly mobile equilibrium with lower polymeric forms. In solutions of 0.1 ionic strength, the β component makes its appearance only after about two-thirds of the carboxyl groups of insulin have been esterified, but, in the presence of 0.2 M salt, this component can be detected early in the esterification process.

The β aggregation may be considered as a beginning of a salting-out effect to which the protein becomes progressively predisposed as its hydrophobic character increases on esterification. While this factor could account for the increasing abundance of the β component during the major phase of the esterification reaction, it has been noted that the relative concentration of the β component continues to increase after esterification of the initially available carboxyl groups is complete (5), this increase being disproportionally greater than the additional methoxyl uptake. In this range, and possibly also in earlier stages, the formation of the β component must be due to a change other than that of the esterification itself. It is possible that such a change entails intramolecular rearrangement and thus represents a form of denaturation. This interpretation is in accord with preliminary viscosity measurements which have shown that the presence of the β component is associated with an increase in the viscosity of the solution. Since the specific viscosity of a 1 per cent solution, containing about three-fourths of the protein in the β form, was still below 0.1 and

the solution was devoid of demonstrable flow birefringence, the shape changes were of considerably lower magnitude than were those involved in the heat denaturation of acid insulin (9) or of insulin methyl ester itself (5). It has been shown in the preceding paper of this series that a methoxyl uptake beyond about 3 per cent is coincident with the deamination and esterification of acid amide groups. It is likely that this chemical change, the progressive β aggregation in this range, and the postulated intramolecular rearrangements are interrelated phenomena.

The results represented by Figs. 5 and 6 and their description in the corresponding text show that the ultracentrifugal behavior at a given pH and salt concentration is a sole function of the methoxyl content. Although the appearance of the β component is to be considered as an undesirable side reaction, this reaction could not be avoided by changes in conditions of esterification. The formation of this component must, therefore, be considered as an integral part of the esterification process.

It is a pleasure to acknowledge our thanks to the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana, for the supply of crystalline zinc insulin, and for the financial support of this work.

SUMMARY

The process of esterification of insulin with acid methanol has been followed by ultracentrifugal analyses of 1 per cent protein solutions of 0.1 and 0.2 ionic strength and pH 3.7. The sedimentation constant of one of the two ultracentrifugal components (α) decreases with increasing degrees of esterification, probably as a result of progressive disaggregation. A limiting value of $S = 1.5$ is approached when methylation of the initially available carboxyl group is complete. A more rapidly sedimenting (β) component appears as esterification proceeds. While the sedimentation rate of this component is unrelated to the methoxyl uptake, its relative concentration increases markedly with increasing ionic strength and increasing esterification. The nature of the aggregation process which leads to the formation of this component has been discussed.

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THE RELATIONSHIP BETWEEN HEXACHLOROCYCLO- HEXANE AND INOSITOL IN NEUROSPORA*

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A metabolic relationship between inositol and the γ isomer of hexachlorocyclohexane (gammexane) has been reported by several investigators. An inositol-requiring strain of *Saccharomyces* has been demonstrated by Kirkwood and Phillips (1), to be inhibited by gammexane. This inhibition was specifically reversed by the addition of sufficient amounts of *meso*-inositol. Buston, Jacobs, and Goldstein (2) have demonstrated a similar effect on the inositol-dependent *Nematospora gossypii*. The action of a purified pancreatic α -amylase which contained inositol has been shown to be inhibited by gammexane and reversed by *meso*-inositol (3). However, crystalline pancreatic α -amylase (4) and crystalline malt α -amylase (5) have been reported not to be inhibited by gammexane. On the other hand, Schopfer and coworkers have shown that gammexane inhibits growth of pea roots in sterile culture (6) and that it inhibits growth of various inositol-independent organisms (7). None of the inhibitions could be reversed by the addition of *meso*-inositol. Fromageot and Cofino (8) were also unable to establish any relationship between these two compounds in regard to the growth of twelve anaerobic and thirteen aerobic inositol-independent bacteria. The purpose of this paper is to report evidence obtained with *Neurospora* that gammexane causes two types of inhibitions and that only one of these is specifically reversed by *meso*-inositol.

Materials and Methods

Two macroconidial strains of *Neurospora crassa* were used in this work, wild type strain SY-7A and *inositolless* mutant 37401A. The experiments were carried out at 30° in growth tubes which contained 10 ml. of minimal medium (9) in 2 per cent agar. Inoculation was made at one end of the tube with a loop of a spore suspension and growth was followed by measuring progress along the tube. Since gammexane is soluble in water only up to 60 γ per ml., but is very soluble in ethyl alcohol, a stock solution was made up in ethanol and added before sterilization to the experimental tubes in the desired concentrations.

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Results

The control growth rate of the wild type strain SY-7A on minimal medium without gammexane was 3.6 mm. per hour. 10 γ of gammexane per ml. produced no significant depression of this rate. However, the addition of 30 and 50 γ per ml. resulted in growth rates of 2.5 and 2.05 mm. per hour respectively. The presence of as much as 100 γ per ml. of *meso*-inositol had no effect on these rates; thus no relationship between *meso*-inositol and gammexane was demonstrable in wild type *Neurospora*.

Strain 37401 was described as an assay organism for inositol by Beadle (10). He noted that on suboptimal levels of inositol in liquid media the

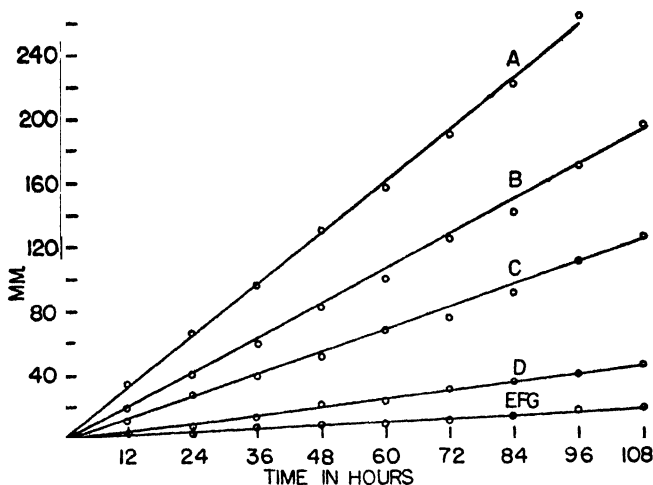


FIG. 1. The effect of various levels of inositol on the rate of growth of the *inositolless* mutant 37401. Curve A, 4 γ per ml.; Curve B, 2 γ per ml.; Curve C, 1 γ per ml.; Curve D, 0.75 γ per ml.; Curves E, F, and G, 0.5, 0.4, and 0.25 γ per ml. (colonial morphology).

mycelia grew in pellets, in contrast to the spreading growth of wild type or of the mutant in the presence of adequate inositol concentrations. It has been shown that a colonial type growth is characteristic of an inositol deficiency. This morphological effect of suboptimal inositol levels is manifested by a marked depression of growth rate to about 0.20 mm. per hour, as shown in Fig. 1.

Strain 37401 was strikingly inhibited by gammexane in concentrations above 10 γ per ml. A typical series of results with strain 37401 on 4 γ per ml. of inositol and 30 γ per ml. of gammexane is shown in Fig. 2. For the first 24 hours growth was retarded considerably from a control rate of 3.0 mm. per hour to 1.5 mm. per hour in the wild type (Curve B) and to 1.6 mm. per hour in the mutant strain (Curve C-1). However,

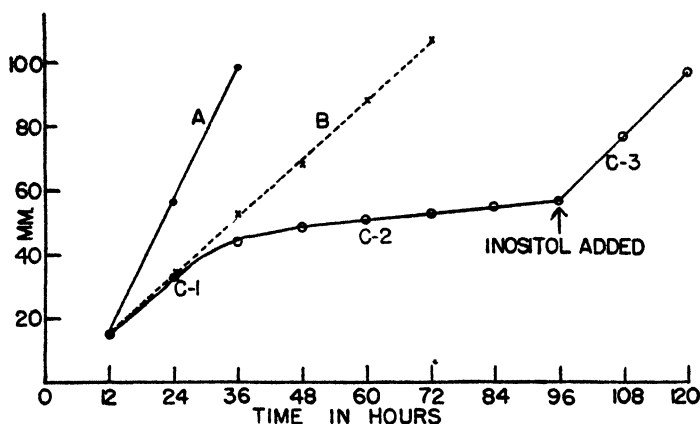


FIG. 2. The effect of gammexane on the wild type and the mutant strain 37401. Curve A, strain 37401 on 4 γ per ml. of inositol, rate 3.0 mm. per hour; Curve B, strain SY-7A (wild type) on 30 γ of gammexane, rate 1.5 mm. per hour. Curve C-1, strain 37401 on 4 γ per ml. of inositol and 30 γ per ml. of gammexane, rate 1.6 mm. per hour; Curve C-2, colonial morphology, rate 0.19 mm. per hour; Curve C-3, addition of 1 mg. of inositol to the growth tube, rate 1.4 mm. per hour.

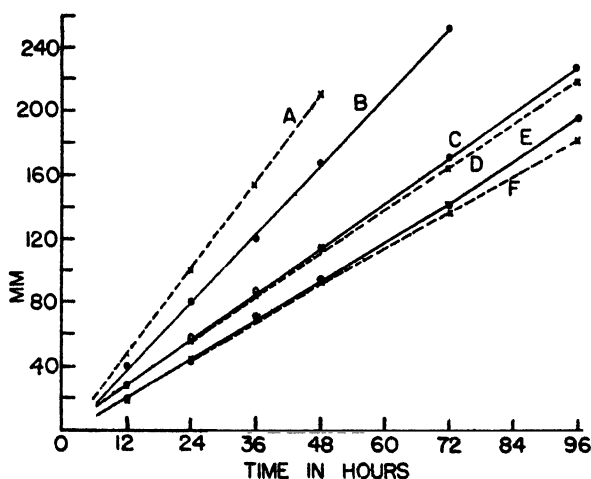


FIG. 3. The effect of gammexane on growth rate of strains SY-7A and 37401 in the presence of excess inositol. 100 γ of inositol per ml. (controls), Curve A, strain 37401, rate 4.6 mm. per hour; Curve B, strain SY-7A, rate 3.6 mm. per hour. 30 γ of gammexane per ml. and 100 γ per ml. of inositol, Curve C, strain SY-7A, rate 2.5 mm. per hour; Curve D, strain 37401, rate 2.5 mm. per hour. 40 γ of gammexane per ml. and 100 γ per ml. of inositol, Curve E, strain SY-7A, rate 2.05 mm. per hour; Curve F, strain 37401, rate 2.00 mm. per hour.

after 36 hours, the growth rate in the mutant strain was sharply reduced to 0.19 mm. per hour with the simultaneous appearance of colonial type

growth (Curve C-2), suggestive of an inositol deficiency. After 94 hours, a sterile solution containing 1 mg. of inositol was added to the surface of the agar. The rate of growth increased to 1.4 mm. per hour and the mycelium ceased to be colonial in appearance. Even with a large excess of inositol the rate did not reach the control of 3.0 mm. per hour of strain 37401 in the absence of gammexane. (Compare Curves A and C-3 in Fig. 2.) The reversed rate was strikingly similar to that of wild type on 30 γ of gammexane. (Compare Curves B and C-3 in Fig. 2.)

These data suggest the existence of two types of inhibition. One would be an inhibition which affects both wild type and the mutant and bears no demonstrable relationship to *meso*-inositol. The other would affect only the mutant and produce colonial growth typical of inositol deficiency. This latter inhibition is specifically and completely reversed by adding excess *meso*-inositol.

If the specific antiinositol effect is truly different from the general inhibition produced in both strains, then the mutant grown on excess inositol and gammexane should behave in a manner similar to the wild type under the same conditions. The experiment summarized in Fig. 3 shows that both the mutant strain on an excess of inositol (100 γ per ml.) and the wild type strain are inhibited equally with several levels (30 and 40 γ per ml.) of gammexane.

DISCUSSION

The present results with an inositol-dependent and an inositol-independent strain of the same organism suggest that gammexane does not demonstrably affect the metabolism of endogenously synthesized inositol but does inhibit the utilization of exogenously supplied inositol. These results reconcile the discrepancies previously reported in the literature, since all positive evidence for the existence of a metabolic relationship between *meso*-inositol and gammexane has been obtained with inositol-requiring organisms (1, 2) and all negative evidence with organisms that have no exogenous requirement for the vitamin (6-8).

At least in *Neurospora* it also seems clear that gammexane has two inhibitory effects. The first is an inhibition apparently independent of inositol metabolism and the second is a competitive inhibition of the utilization of the vitamin by an inositol-dependent organism. Although the mechanism of the specific antiinositol action of gammexane is not entirely clear, there are two possible ways in which the mutant strain and the wild type organism may differ in their response to this antime-tabolite. One possibility is that gammexane only interferes with the metabolism of exogenously supplied inositol in the mutant, without interfering with the normal intracellular biosynthesis, or the utilization of

endogenous inositol in the wild type. Alternatively, gammexane may interfere with inositol metabolism in both strains. In the inositol-requiring organism the available inositol can be limited to a level which is optimal for growth but still allows an inhibitory ratio of gammexane to inositol to be reached. Therefore a specific inhibition by gammexane can be shown in the mutant strain. In contrast, in the wild type strain, because of its capacity for continued synthesis of inositol, the critical ratio cannot be attained and no inhibition can be experimentally demonstrated.

SUMMARY

1. Evidence is presented that the γ isomer of hexachlorocyclohexane inhibits the growth of both the wild type *Neurospora crassa* and of an inositol-requiring mutant strain.

2. An inhibition not reversible by inositol has been demonstrated in both strains, and a specific inhibition reversed by inositol has been shown in the mutant strain.

3. It is suggested either that in *Neurospora* gammexane inhibits the utilization of exogenous inositol but not that of endogenous inositol or that the utilization of inositol is inhibited in both cases, but can be demonstrated only when inositol can be limited as in the mutant strain.

4. Similar relationships in other organisms would reconcile the discrepant results in the literature on the specificity of the gammexane-inositol relationship.

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THE INFLUENCE OF TEMPERATURE AND pH UPON THE RATE OF DENATURATION OF RICIN*

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For many years distinction has been made between acid denaturation, alkali denaturation, and the heat denaturation of proteins. In a recent review (24) heat is classified as a "physical" denaturing agent and hydrogen and hydroxyl ions as "chemical" denaturing agents. Nevertheless, it is clear that the intensity factors associated with acidity and alkalinity (the pH) and with heat (the temperature) are continuous and not mutually exclusive functions in aqueous solutions.

The rate of denaturation of a protein in solution is a simultaneous function of temperature and pH. The temperature function has been handled in terms of the Arrhenius equation or some interpretation of it (*cf.* Eyring and Stearn (9)), but the pH function has been dealt with rationally only in limited situations. Steinhardt (27) in a paper on the inactivation of pepsin showed inverse proportionality of the rate constant to the fifth power of the hydrogen ion activity. By assigning five successive ionizations of hydrogen ion to pepsin and ascribing to the product of the fifth of these a unique instability he derived an equation which accommodated the facts. Later Neurath *et al.* (24) demonstrated the applicability of the Steinhardt method to some data on the denaturation of hemoglobin (Cubin (7)) over a pH range of 0.5 unit in which the rate was proportional to the square of the hydrogen ion activity. Lewis (21) suggested that protein denaturation was catalyzed by hydrogen and hydroxyl ions. The rate should then be directly proportional to the hydrogen ion activity on the acid side of a minimum and inversely proportional to it on the alkaline side. Since these expectations are not fulfilled, the suggestion need not be considered further.

From the postulates of Steinhardt (27), suitably generalized and modified, we have developed equations which satisfactorily describe data on the denaturation of ricin from pH 1 to 12 and over a range of rates changing

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by a factor of 10^6 . The inactivation of pepsin as studied by Steinhardt and the denaturation of hemoglobin in the acid range as studied by Cubin (7) and in the neutral zone studied by Lewis (21) are all described by equations based on the same postulates.

The temperature dependencies of the parameters of the equations are described for data obtained at five temperatures between 44–86°. The problems posed by the high temperature coefficients of protein denaturation rates are clearly defined by the method of treatment and some deductions may be drawn from the data about protein structure and its change on denaturation.

Salt concentration is a significant variable in protein denaturation (22, 27). All of the data reported below were obtained at a nearly constant ionic strength of 0.2. This placed a limitation on the acid branch of the study because the pH could not be less than 0.7. The pH of a solution is a function of temperature and all of the pH values reported are for the temperature of reaction. Known temperature coefficients for the various buffers were used in obtaining the corrections as described below.

The criterion of denaturation used is insolubility under conditions of initial solubility, as suggested by Wu (28). This study does not require that the product resulting from denaturation be homogeneous except with respect to essentially zero solubility under the test conditions. There is no direct evidence in the material described below on the question of partial or complete denaturation (1, 24).

The reaction rate followed the first order equation at all temperatures and pH values, and the rate constant is stated here in reciprocal seconds.

Methods and Materials

Procedure—12 ml. of the buffer to be used were prepared by mixing appropriate quantities of solutions of the acid and base forms, each at an ionic strength of 0.4 (KCl was added to give this ionic strength if necessary). The buffer solution was prepared in a 25 × 200 ml. test-tube which was stoppered loosely and immersed for most of its length in a constant temperature oil bath. 10 ml. of a stock protein solution were placed in a similar tube in the bath. If the temperature was low enough, no appreciable change occurred in the stock solution and it was allowed to come to bath temperature. To start the reaction, 10 ml. of the buffer solution were blown from a pipette into the protein solution, followed by a stream of air bubbles. A stop-watch was started at the half delivery time of the pipette. If the protein denatured at an appreciable rate in the stock solution, the buffer solution was heated to a temperature above the bath temperature and added to the stock protein solution before the latter reached bath temperature. Temperatures were so chosen that the

final mixture was close to the bath temperature. Small errors in the zero time and in the temperature on mixing are minimized by the graphical method used in obtaining the first order rate constant. As soon as possible after mixing and at suitable intervals thereafter 1 ml. samples of the mixture were pipetted into 1 ml. portions of acetate buffer at pH 5 (stopping buffer). The residual soluble protein in the solution was estimated after centrifuging and from the series of figures and the corresponding sampling times a first order constant was obtained. The pH of the residual reaction solution after all samples had been taken was measured with a Coleman pH meter and glass electrode at room temperature.

Protein—The protein used was crystalline ricin (19) prepared in this laboratory.¹ It had been recrystallized four to six times by solution in dilute hydrochloric acid followed by neutralization of the acid. The crystalline material was kept as a paste in the cold room under toluene. A quantity of the paste (1 to 1.5 gm.) was dissolved in water with the aid of 1 or 2 drops of 0.1 M HCl and diluted to 50 ml. for each day's work. The concentration of protein was not exactly controlled from day to day because it does not enter into the calculation of the first order constant. Independent experiments showed that the constant was not dependent to any significant extent upon the protein concentration. The stock solution was essentially free of electrolytes and its contribution to the ionic strength of the final mixture was ignored. The pH of the stock solution was between 4.0 and 4.5.

Buffers—Because of the wide range of pH covered in the experiments, several buffer systems were necessary. For each acid-base pair solutions having ionic strengths of 0.4 were prepared by adding potassium chloride to make up the ionic strength in the case of the unionized member of the pair. Only potassium compounds were used in the preparation of the solutions in order to avoid the large errors produced by sodium ions at the glass electrode. The solution pairs used included HCl-KCl; glycine, KCl-HCl; acetic acid, KCl-KOH; KH_2PO_4 - K_2HPO_4 ; boric acid, KCl-KOH; KHCO_3 - K_2CO_3 ; and KCl-KOH.

Analysis—In conformity with the operational definition used for denaturation, the analysis consisted of the estimation of the proportion of the protein remaining soluble in an acetate buffer at pH 5 containing 0.5 M sodium acetate and 0.3 M acetic acid. The mixtures of sample and stopping buffer (1 ml. of each) were allowed to stand for at least an hour to complete the coagulation of the denatured protein and centrifuged. A fixed volume (about 0.5 ml.) of the supernatant was removed with a constriction pipette (20) and added to 8.5 ml. of water containing 1 mM sodium carbonate. 1 ml. of a 5:1 dilution of the Folin-Ciocalteu phenol

¹ Cannan, R. K., and Benaglia, A. E., in preparation.

reagent (10) was added and the solutions mixed by inversion. After 20 minutes or more the colored solutions from a single run were read in a Klett-Summerson colorimeter with a filter having maximum absorption at 660 $m\mu$. The readings are proportional to the concentrations of protein over the range used if the instrument is set with an appropriate blank.

Calculation of Velocity Constants—The logarithms of the colorimeter readings were plotted against the time and a straight line drawn through the points. An occasional experiment, especially at the higher velocities, was rejected when it was found that the data did not fit a line. From the slope of the line multiplied by 2.303 the velocity constant in reciprocal seconds was obtained. Usually nine or ten points were taken for each run.

Determination and Corrections of pH—If necessary the pH measured at the glass electrode was corrected for the alkaline error according to the data of Jordan (8, 17). The pH was then adjusted to the actual temperature of the experiment. This was done by using the data of Harned and coworkers for the temperature variation of the dissociation constants (12, 13, 15). The temperature variation of the pH of the buffers was assumed to be the same as for the pK except in the instances in which hydroxyl ion concentrations were significant. In these instances the variation in pK_w was also taken into account by calculating the pH values for constant composition at different temperatures. The corrections were then taken from these curves. Systematic errors such as small amounts of impurities in the buffers and liquid junction potentials will not affect the temperature corrections significantly.

Temperature Control—The oil bath showed temperature variations less than $\pm 0.02^\circ$ at the lower temperatures and up to $\pm 0.1^\circ$ at higher temperatures.

Timing—The stop-watch used read to 0.1 second, but no effort was made to read it more precisely than 1 second. About 1 second was occupied in the transfer from the reaction mixture to the stopping solution. With practice a first sample was obtained in 15 to 20 seconds after mixing and thereafter 8 to 10 second intervals sufficed for sampling.

Data and Analysis

The data are plotted in Fig. 1 to show the relationship between pH and the logarithms of the first order constant ($\log j$) at each temperature. The limitations set by technical difficulties in handling either very slow or very fast reactions by the methods employed and the limitation of ionic strength of 0.2 are obvious.

Relationship of pH and Rate—The lines drawn on Fig. 1 for each temperature are derived from the equations developed below. These equations are based on two simple postulates. As stated above, their applica-

bility in limited regions has been previously demonstrated. A general equation based on the postulates is demonstrated below, but the working equations used for estimating the parameters are more readily comprehended and will be developed first.

The postulates are as follows: (1) The protein is a polyvalent acid and base (in the Brønsted sense) which by association or dissociation of protons may assume many ionic forms, the activities of which are related to one another by mass action equations involving (H^+); (2) a number of the prototropic changes are accompanied by changes in the stability of the

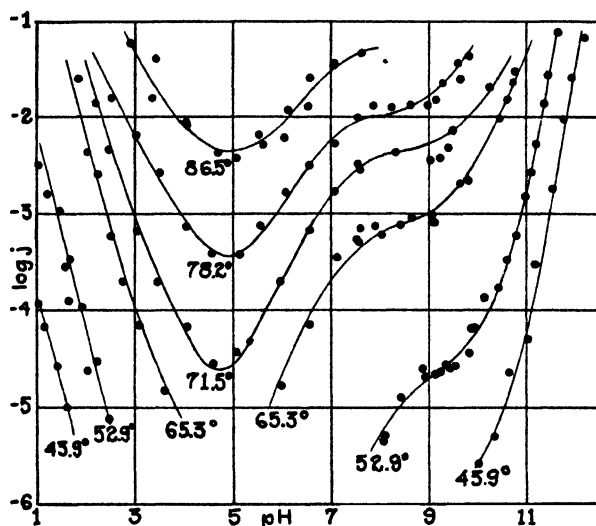
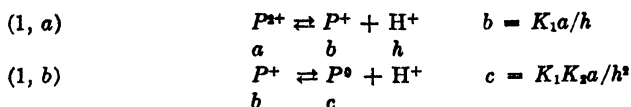


FIG. 1. The first order velocity constant j as a function of pH. The points are experimental. The lines are drawn from the constants of Table I and the equations in the text.

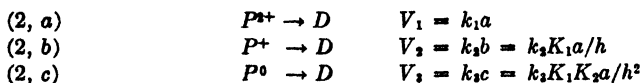
protein which are reflected in the rate of production of an insoluble product. The stability may be increased or decreased by dissociation of protons. The number of prototropic steps associated with the change in stability can be estimated in favorable regions from the slopes of the straight lines which show in the plot of the logarithms of the first order constants ($\log j$) against pH. This was recognized by Steinhardt (27), but the limitations of his data prevented him from demonstrating any significant change in stability except for the fifth proton dissociation in a series of five.

Acid Region—The most acid form of the protein will be arbitrarily assigned two significant dissociation steps and written P^{2+} . No difference except a greater complexity of exposition results from using P^{2+} as the initial form. In the equations and formulae the lower case letters represent the concentrations or activities of the molecular species symbolized

directly above them in the chemical equations. The proton dissociations are written as follows:



Each of the molecular species P^{2+} , P^+ , and P^0 is assigned a unique velocity (V_n) of transformation into denatured protein (D)



If the total protein is A and x is the amount transformed to D , then

$$(3) \quad A - x = a + b + c = a(1 + K_1/h + K_1 K_2/h^2)$$

The total rate of formation of D at any time is

$$(4) \quad dx/dt = V_1 + V_2 + V_3 = a(k_1 + k_2 K_1/h + k_3 K_1 K_2/h^2)$$

Division of Equation 4 by Equation 3 and integration of the result shows that the first order constant (at constant pH) is

$$(5) \quad j = \frac{k_1 h^2 + k_2 K_1 h + k_3 K_1 K_2}{h^2 + K_1 h + K_1 K_2}$$

By the usual convention $K_1 > K_2$. If $h < K_2$, Equation 5 becomes

$$(6) \quad j \cong k_1 h^2 / K_1 K_2 + k_2 h / K_2 + k_3$$

Equation 6 describes the acid branches of the curves of Fig. 1. Approximate evaluation of the parameters may be obtained by noting that the terms become successively dominant as pH changes. In the most acid regions

$$(7) \quad j \cong k_1 h^2 / K_1 K_2 \quad \text{or} \quad \log j = \log k_1 / K_1 K_2 - 2 \text{ pH}$$

The extreme acid ends of the curves of Fig. 1 (at 43.9°, 52.9°, 65.3°, and 71.5°) reach a slope of -2 and from the intercept along this slope $\log k_1 / K_1 K_2$ may be evaluated at each temperature. An approximation of $\log k_2 / K_2$ may be obtained in that region of the curves with a slope of -1 , for at some intermediate value of h Equation 8 holds.

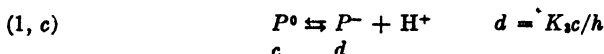
$$(8) \quad j = k_2 h / K_2 \quad \text{or} \quad \log j = \log k_2 / K_2 - \text{pH}$$

When h is sufficiently small $j = k_3$. However, in the final evaluation of k_2 / K_2 and of k_3 successive approximations are necessary to fit Equation 6 and those parts of Equation 9 which are pertinent. In general a three term equation in h is necessary to fit the data in any particular region. The power of h in the predominant term in a given region will be indicated

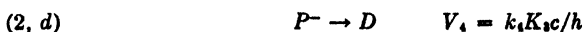
by the slope in Fig. 1 for that portion in which the slope is integral. When several ionic forms make significant contributions to the rate, several terms of the equation must be used. The final values of the parameters from which the curves of Fig. 1 were drawn are entered in Table I.

The condition in Equation 5 that $h \cong K_2$ causes the slope in a plot of $\log j$ against pH to pass from -2 to -1 and when $h > K_1$ the slope passes from -1 to 0 . In actuality a slope of -2 is maintained up to pH 1 and we can conclude that $pK_2 < 1$. Thus pK_2 as well as the smaller pK_1 is less than 1 and cannot, therefore, belong to the carboxyl groups whose pK values in proteins appear in the region of pH 2 to 4.

Middle Range—Between pH 5 and 9 the pertinent equilibrium is



and the rate equations are Equation 2, *c* above and



From Equations 1, *c*, 2, *c*, and 2, *d*, Equation 9 is obtained.

$$(9) \quad \frac{k_2 h + k_4 K_2}{h + K_2}$$

When $h > K_2$, $j = k_2 + k_4 K_2/h$. At the acid end of the region $j = k_2$ (the minimum rate after correction for the contributions of V_2 and V_4). As h decreases, the second term becomes larger and $j \cong k_4 K_2/h$ or $\log j = \log k_4 K_2 + \text{pH}$. The predicted slope of $+1$ is realized at several temperatures in Fig. 1 and from it $\log k_4 K_2$ can be evaluated as the intercept on this slope. When $h \ll K_2$ in Equation 9, $j \cong k_4$. This constant can be evaluated as the value of j on the plateaus of the curves of Fig. 1. When $h = K_2$ in Equation 9, $j = (k_2 + k_4)/2$. Since $k_4 \gg k_2$, this means practically that $\text{pH} = pK_2$ at a point on the curves where $\log j$ is 0.3 less than $\log k_4$. The preliminary estimates of the three parameters (k_2 , K_2 , k_4) are revised in accordance with the fitting of the data. Final values are entered in Table I. Although K_2 is in the region ordinarily ascribed to the amino group in proteins, its variation with temperature is so unusual (see below) that its actual assignment to proton dissociation from an amino group seems doubtful.

Alkaline Region—In this region the equilibrium equations required are

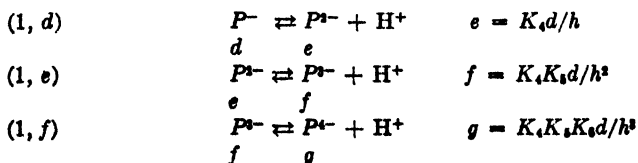
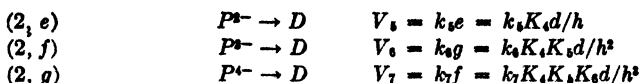


TABLE I
Parameters for Denaturation of Ricin at Various Temperatures

Parameter	43.9°	52.9°	65.3°	71.5°	78.2°	86.5°
k_1/K_1K_2	0.017	0.69	60	316		
k_2/K_2			0.056	0.63	6.8	44
k_3				$<1 \times 10^{-6}$	2×10^{-4}	3.2×10^{-3}
k_4		2×10^{-5}	8.3×10^{-4}	4.8×10^{-3}	1.2×10^{-3}	5.6×10^{-3}
K_3		3.3×10^{-9}	3.0×10^{-8}	4.6×10^{-8}	9×10^{-8}	1×10^{-7}
k_5K_4	2.5×10^{-16}	4.6×10^{-15}	2.2×10^{-14}	7.95×10^{-13}	5.6×10^{-12}	
$k_6K_4K_5$						
$k_7K_4K_5K_6$	3.2×10^{-48}	1.0×10^{-36}	5.6×10^{-24}			

Dimensions, k in reciprocal seconds, K in moles per liter.

The rate equations are Equation 2, *d* above and



Combining as before gives

$$(10) \quad j = \frac{k_4h^3 + k_5K_4h^2 + k_5K_4K_5h + k_7K_4K_5K_6}{h^3 + K_4h^2 + K_4K_5h + K_4K_5K_6}$$

if $h > K_4$

$$j = k_4 + k_5K_4/h + k_5K_4K_5/h^2 + k_7K_4K_5K_6/h^3$$

With the same approximations as before the successive terms suggest that the plot of $\log j$ against pH should show successive slopes of 0, 1, 2, and 3. Such slopes are found at different temperatures. In the alkaline branch of the 43.9° curve it was not necessary to include the h^2 term to fit the data. This means that $k_7K_6h \gg k_6$ (or k_6 is zero). As the pH approaches pK_4 , the slope in Fig. 1 should decrease from 3 according to Equation 10. Since this does not occur up to pH 12 at 43.9°, pK_4 must be greater than 12.

The use of numerous numerical constants to rationalize the data may be criticized. In justification it may be noted that the ranges of the independent variable (h) and the dependent variable (j) are unusually large. These ranges, 10^{11} and 10^5 respectively, are covered by equations developed from what seems to us the simplest and most direct of possible postulates. Further rationalization in terms of the theory of absolute reaction rates (11) is developed in a later section.

General Formulation

All the equilibrium Equations 1, *a* to *f*, and rate Equations 2, *a* to *g*, can be put into a single equation by the same algebraic manipulation used in limited regions. Each regional equation as used in the preceding section becomes a special case dependent on the position of the numerical value of h in the conventional series $K_1 > K_2 > K_3 \dots > K_m$ when the total number of acid forms considered is m . There are then $m + 1$ ionic species of the protein which undergo denaturation with rate constants $k_1, k_2, k_3 \dots k_{(m+1)}$. The successive products of the dissociation constants may be written $\prod_{m=0}^{i-1} K_m$ (for the *i*th term) with the convention that $K_0 = 1$. The general formulation is then

$$(11) \quad j = \frac{\sum_{i=1}^{m+1} k_i \left(\prod_{m=0}^{i-1} K_m \right) h^{m+1-i}}{\sum_{i=1}^{m+1} \left(\prod_{m=0}^{i-1} K_m \right) h^{m+1-i}}$$

In the range of data given above for ricin $m = 6$. The identification of c (Equation 1, b) as having zero charge is completely arbitrary and no implication of an isoelectric form is intended. The minimum rate observed at about pH 5 is a reflection of the rise and fall of the k values for the various ionic species in the significant dissociations.

When $m = 5$ and $h > K_1$, there results the relationship used by Steinhart (27) to describe the inverse fifth power dependence of pepsin inactivation. As he recognized, the falling away from a slope of 5 on the plot of $\log k$ versus pH at the higher pH values indicated approach to pK_1 . When $m = 2$ and $K_2 \gg h$, the first term indicates the dependence of j on the square of the hydrogen ion concentration, as observed by Neurath *et al.* (24) in the data of Cubin (7) for hemoglobin. When $m = 2$ and $K_1 \gg$

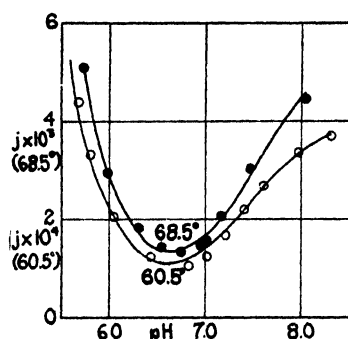


FIG. 2. Denaturation of hemoglobin. The first order velocity constant j as a function of pH. The lines are calculated from the constants in Table II and Equation 9 in the text. The points are experimental from Lewis (21).

$h > K_2$, the data of Lewis (21) for hemoglobin denaturation at 60° and 68° are accommodated (see Fig. 2). The equation developed is

$$(12) \quad j = \frac{k_1 h}{K_1} + \frac{k_2 K_2}{h + K_2}$$

because k_2 is substantially zero. The lines in Fig. 2 were drawn for this equation and the parameters in Table II. The points are experimental as recorded by Lewis (21). Table II also gives the equilibrium and velocity reactions which are pertinent to Equation 12.

Significance of Mixed Constants

The parameters k_1/K_1K_2 , k_2/K_2 , etc. (Table I), are mixed constants because they refer formally to reversible and irreversible steps at the same time. It seems best to consider these in terms of the theory of absolute reaction rates as set forth by Glasstone, Laidler, and Eyring (11). In this

system the rate constant is converted into an equilibrium constant and a frequency. The rate constant $k = (kT/n)K^*$ where k/n is a universal constant, T is the absolute temperature, and K^* is the equilibrium constant between normal molecules entering into the reaction and the activated molecules. Thus for a reaction $A + B \rightarrow M^*$, $K^* = [M^*]/[A][B]$. M^* signifies the active complex. If the reaction is unimolecular, K^* gives the constant ratio between activated and non-activated reactants, pre-

TABLE II
Denaturation of Hemoglobin; Rate Data from Lewis (21)

Reaction	Constant	Numerical values		
			60.5°	68.5°
$P^+ \rightleftharpoons P^0 + H^+$	K_1			
$P^0 \rightleftharpoons P^- + H^+$	K_2	k_1/K_1	210	2820
$P^+ \rightarrow D$	k_1	k_2	0	0
$P^0 \rightarrow D$	k_2	k_3	4.2×10^{-4}	5.9×10^{-3}
$P^- \rightarrow D$	k_3	K_2	4.0×10^{-3}	3.0×10^{-3}

Dimensions, k in reciprocal seconds, K in moles per liter.

TABLE III
Relationship of Mixed Parameters to Activation Equilibrium Constants

Parameter	Constant	Activating reaction	Equation No.
k_1/K_1K_2	K_1^*/K_1K_2	$P^0 + 2H^+ \rightleftharpoons P^{*+}$	3, a
k_2/K_2	K_2^*/K_2	$P^0 + H^+ \rightleftharpoons P^{*+}$	3, b
k_3	K_3^*	$P^0 \rightleftharpoons P^*$	3, c
K_3	K_3	$P^0 \rightleftharpoons P^- + H^+$	3, d
k_4	K_4^*	$P^- \rightleftharpoons P^{*-}$	3, e
k_5K_4	$K_5^*K_4$	$P^- \rightleftharpoons P^{*-} + H^+$	3, f
$k_7K_4K_5K_6$	$K_7^*K_4K_5K_6$	$P^- \rightleftharpoons P^{*-} + 3H^+$	3, g

sumably related to the chance of accumulation of sufficient energy by thermal transmission to a particular molecule to bring about reaction.

The parameter k_1 of Equation 2, a is thus identical with $(kT/n)K_1^*$, and if we divide k_1/K_1K_2 by (kT/n) , the result K_1^*/K_1K_2 is a ratio of equilibrium constants. It then becomes formally clear what reactions and standard states are referable to the constants of Table I. The reactions corresponding to the mixed constants are listed in Table III.

The rôle of the proton in the formation of the "activated complex" is clearly shown in these equations. Only K_3 refers to a non-activating prototropic change. The formal difference between Equation 3, a to g, and

Equations 1, *a* to *f*, and 2, *a* to *g*, is in the separation of prototropic change and thermal activation in the latter case, whereas in the former the two are regarded as simultaneous processes. The K_3 process is the same for both and is definitely not to be considered an activating proton dissociation.

Thermodynamics of Activation Processes; Temperature Dependence

The processes of activation in the theory of absolute reaction rates are analyzed in terms of thermodynamic analogues ΔH^* , ΔS^* , and ΔF^* . These are obtained by plotting the logarithms of K^* against $1/T$. Such plots are shown in Fig. 3 for each of the constants of Table III. Apart from

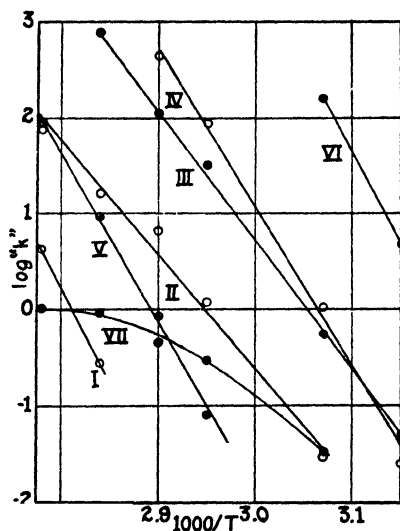


FIG. 3. Denaturation of ricin. The activation equilibrium constants as a function of temperature. The value of $\log k$ for each line is (Curve I) $18 + \log K_3^*$, (Curve II) $18 + \log K_4^*$, (Curve III) $\log K_3^* K_4$, (Curve IV) $15 + \log K_1^*/K_1 K_3$, (Curve V) $15 + \log K_2^*/K_2$, (Curve VI) $53 + \log K_7^* K_4 K_5 K_6$, (Curve VII) $7 - pK_3$.

that for $\log K_3$, the plots fall on straight lines, indicating that ΔH^* and ΔS^* are essentially independent of temperature (within the range of the data). The values of ΔH^* and ΔS^* are obtained from the slopes and intercepts of these lines and are entered in Table IV; ΔF^* (338°) is calculated from them.

The prototropic constant, K_3 , has unusual thermodynamic properties. The data of Table I show that K_3 varies markedly with temperature. Its variations fit the equation $pK_3 = 6.99 + 0.00143 (T - 358)^2$. The calculated values of pK_3 differ from the experimental by a maximum of 0.09 unit and on the average by 0.03 unit. The fit may be judged from Curve VII of Fig. 1. The form of the equation is that used by Harned and

Owen (13) to describe the behavior of many acids.³ However, the coefficient of the second term is ordinarily 0.00005; in one case, boric acid, it is 0.00006. The coefficient required by our data is 30 times as great and we take this to indicate that the group concerned cannot be identified by analogy of the pK values. From the equation, pK₂ is 6.99 at 85° and 12.84 at 25°, at which most pK data are available.

TABLE IV
Thermodynamic Analogues of Activation Reactions Obtained from Fig. 3

Equation No.	Constant	ΔH°	ΔS°	ΔF_{298}°
		kilocalories	calories per °C.	kilocalories
3, a	K_1°/K_1K_2	79	173	20.5
3, b	K_2°/K_2	81	161	26.4
3, c	K_3°	89.3	168.5	32.3
3, d	K_2 (338°)	30.0	54	11.7
3, e	K_4°	53	41.6	38.9
3, f	$K_5^\circ K_4$	62.7	58.2	42.8
3, g	$K_7^\circ K_1 K_2 K_4$	86.4	32.6	75.4

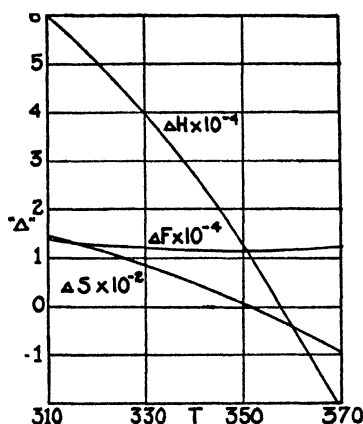


Fig. 4. The thermodynamic functions of Equation 3, d as functions of temperature

From this equation the thermodynamic functions of the prototropic Equation 3, d become

$$\Delta F = -RT \ln K_2 = 2.303RT \text{p}K_2 = 0.00654T^3 - 4.685T^2 + 870.6T$$

$$\Delta S = -d(\Delta F)/dT = -0.01963T^2 + 9.370T - 870.6$$

$$\Delta H = \Delta F + T\Delta S = -0.01309T^3 + 4.685T^2$$

A graphical presentation of the variation of these functions appears in

³ The form has been abandoned for more exact but less rigid series approximations (14). For all practical purposes, however, the formulation is excellent.

Fig. 4. Their unusual dependence on temperature is evident. The very large entropy increase associated with the proton dissociation in the range of our measurements is evidence for a considerable release of structural rigidity accompanying the reaction. The considerably lower entropy of activation, starting from P^- as the standard state (Equations 3, e, 3, f; and 3, g) instead of from P^0 as the standard state (Equations 3, a, 3, b, and 3, c), shows that the formation of P^- from P^0 is in the direction of activation.

In the usual analysis of dependence of denaturation rate on temperature, the logarithms of the rates at constant pH are plotted against the reciprocals of the absolute temperatures. In our data this gives satisfactory fitting in the acid and alkaline regions. In the neighborhood of pK_3 , however, the lines break into two portions and the "energy of activation" becomes ambiguous. This finding is not uncommon for denaturation and inactivation. In the analysis presented above, the "break" appears as a variation of pK_3 with temperature. The problem then becomes one of describing the chemical nature of the group associated with pK_3 .

DISCUSSION

Ricin, aside from its toxicity, has given no indication of being an abnormal protein. Its amino acid composition and acid-base titration curves are in approximate agreement.³ We therefore feel free to generalize on the basis of the denaturation study above, especially since the indications are that in several cases (pepsin, hemoglobin) application of the same kind of analysis gives the same general picture.

In globular proteins all the prototropic groups of the amino acid side chains are free for acid-base titration (6). Ricin contains about 50 such groups in the general range of pH covered by the data above. Only a few (six) apparently have prime significance for denaturation and some of these are not the well recognized titrating groups of proteins. Of those included here, K_1 and K_2 are too acid for carboxyl groups, K_3 has an unusual temperature characteristic, but $K_4 \dots K_6$, each of which is less than 10^{-12} , are possibly attributable to guanidinium groups.

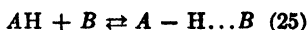
If we assume, with many others, that the native character of globular proteins is a matter of a specific geometric configuration imposed upon a polypeptide chain (or a covalently linked group of such chains), we are constrained to consider the kinds of bonds holding the structure together. It has generally been posited that these are of two kinds, electrostatic or salt bridges dependent on the electrostatic attractions between positively and negatively charged groups in the side chains of the amino acids, and those dependent on the residual valence of hydrogen attached to negative

³ Cannan, R. K., and Benaglia, A. E., unpublished data.

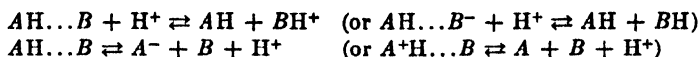
atoms such as oxygen, nitrogen, and sulfur. These bonds are the hydrogen bonds. Only a few of the prototropic changes known to occur during the titration of a protein between pH 1 and 12 appear to have any significant effect on the stability of the native structure, as shown by the relationship of pH and denaturation rate. Salt bridges which are obviously broken as —NH_3^+ loses H^+ and COO^- acquires H^+ must then be of minor importance for the native molecular configuration of globular proteins.

Intramolecular hydrogen bonds (chelations) have been postulated between the —C=O and —NH— of successive peptide bonds (18) as well as between adjacent peptide chains (16). However, the folding of a peptide chain appears to be stable and it would seem that a common or universal structure of proteins would result from the operation of such bondings (5, 23). Bull (5) and Neurath *et al.* (23) have pointed out that it is only by utilizing the specific chelating possibilities of the side chain groups that a specific molecular architecture is likely to result. Our results seem more readily interpretable on the basis of specific chelations between groups in the side chains of proteins rather than on the basis of the general polypeptide chain structure and chelations within its limits. The latter are perhaps adequate for the fibrous proteins.

In the formation of a hydrogen bridge an acceptor and a donor group are involved. In the reaction of hydrogen bond formation



the strength of the bond is increased the more positive A is and the more negative B is. The effect of hydrogen ion concentration on such a bond might be indicated by the reactions



Chelation within a compound should affect its strength as an acid and in turn hydrogen ion concentration should affect the extent of chelation. The former has been noted in the case of salicylic acid (2) and diortho-hydroxybenzoic acid (3) as compared with *m*- and *p*-hydroxybenzoic acids; the reverse follows from the principles of equilibrium.

We believe the prototropic groups implicit in the relationship between pH and denaturation rate are prototropic chelations. For ricin six such bonds are indicated. These involve six donor and six acceptor groups. There may be some duplication among these and there probably are additional chelations not affected by hydrogen ion, or which are outside the range of our data.

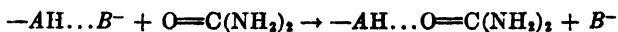
The interpretation of the high temperature coefficients of protein denaturation rates in terms of the thermodynamic analogues ΔF^* , ΔH^* , and ΔS^* is discussed by Glasstone, Laidler, and Eyring (11). The high values

of ΔS^* found are taken as a measure of the large reduction of the rigidity of structure in the activated state compared to the normal state. Comparison of ΔS^* for Equations 3, *a*, 3, *b*, and 3, *c* indicates that the loss in rigidity is essentially the same in these processes. If we can assume that the differences between the activated states P^{*2+} , P^{*+} , and P^{*0} are the same as those between the corresponding non-activated species P^{2+} , P^{+} , and P^0 , then the postulated breaking of prototropic chelations does not of itself give rise to a marked loosening. The structure is weakened in that the acquisition of less thermal energy is necessary to break the structure, and the factor of safety has been decreased by the loss of the bond. ΔF^* becomes less the more such bonds are broken, presumably by 5.9 kilocalories per bond. If all of the free energy of activation is ascribed to such bonds, then six are broken in activating P^0 , five in activating P^{-} , and four in activating P^{2-} . The entropy associated with each of the six bonds would be 25 e.u. Evidently the bonds are so interlaced that the specific structure resulting from them is not readily lost until most of them have come loose.

The behavior of Equation 3, *d* exemplifies what would be expected of the breaking of a prototropic chelation which results in a loss of structural rigidity *per se*. ΔS for the proton dissociation is large and ΔS^* of activation of the product, P^{-} , is very much less than the ΔS^* for P^0 . Equation 3, *d* may be identical with that sometimes found in ultracentrifugal studies of proteins, which is expressed as a marked change in shape with pH (26). At any rate the actual loosening of structure accompanying Equation 3, *d* at 65° is reflected in the lower entropies of activation in the alkaline ranges.

The chelations of the native protein presumably originate at the time of synthesis of the protein by a biological agent. If six pairs of acceptors and donors are involved, there are 720 possible ways of setting up pairs of these. Some will involve such severe strains that the structures will be impossible, and some will have a degree of metastability allowing them to survive; among these must be sought the biologically significant structures. Others of the possible patterns of arrangements are the more probable ones of the denatured proteins. Within the native patterns it is possible to imagine limited regions of greater metastability than the entire pattern. Such limited regions might guide the folding back to the native form, even in the absence of the biological molding agent. Thus denaturation may be reversible. On more severe treatment or when less care is used, the reversibility is lost. Reversibility of protein denaturation differs from ordinary chemical reversibility in this respect: the position of the reaction is not completely independent of the intermediate steps in reaching a final state.

In terms of the hypothesis of specific chelation as proposed in this paper the bonds broken by urea, guanidine, etc., are the same as those broken in other types of denaturation. These substances, which contain strong donor and acceptor groups, will compete with normal internal bonds as shown in the equation as follows:



On removing the urea by dialysis, no guide (23) is available to return the protein to the native structure and chelations are reformed randomly (that is, with only the thermodynamic limitations and without biological guidance to a thermodynamically metastable structure). Denaturation by this type of reagent is characterized by low and sometimes negative temperature coefficients (24). It differs from denaturation by thermal energy in the specific direction of the energy of reaction (hydrogen bonding with urea, etc.) into breaking the chelate bonds. Thermal energy should be absorbed in the protein molecules through many degrees of freedom, only a few of which involve hydrogen bonds. The urea and guanidine reactions are specifically directed toward the bonds to be broken.

Denaturation by surface spreading results from the removal of characteristic chelations as a result of the geometrical forces introduced by the surface. It too should have a zero or negligible temperature coefficient.

The failure of certain groups, such as $-SH$, phenol, and $-S-S-$, in proteins to react in their usual way has been ascribed to their mechanical inaccessibility. They were considered to be "inside" the protein molecule and only became "exposed" on denaturation. It would seem more logical to consider these as being bound in hydrogen bridges, for which SH and OH are donors and $-S-S-$ is an acceptor group. This has the virtue of removing the anomalous molecular surface as a barrier to reagents. Crammer and Newberger (4) have already pointed out the impossibility of considering a protein molecule as having regions from which such ions as OH^- are barred. The inactivity in the native protein of the groups mentioned above is recognized as relative; sufficiently strong $-SH$ reactants will bring out $-SH$ groups which do not react with nitroprusside. This is in accord with the hypothesis that the SH groups are engaged in chelate bridges in the native protein.

SUMMARY

The denaturation of crystalline ricin follows first order kinetics. The velocity constants have been determined over a wide range of pH and temperature. By assuming that unique proton dissociations lead to ionic forms which undergo processes of denaturation at specific rates, equations describing the pH dependence of the first order constants are obtained.

(The general form of the equations is applicable to data for pepsin and hemoglobin found in the literature.) The parameters of the equations are obtained from the data and are shown to have the relationships to temperature required by the theory of absolute reaction rates. The thermodynamic analogues of ΔH , ΔF , and ΔS for the activation processes are obtained from the data.

The fact that of the many proton dissociations demonstrable in ricin only six need be considered in fitting the data minimizes the importance of salt bridges in determining the native protein structure. Of these six at least three are not identifiable with any titrating group of the protein. Two are too acid for carboxyl groups and one has a pK with a much more marked temperature dependence than any known prototropic group. It is proposed that the six groups are proton-sensitive hydrogen bonds involved in specific biologically imposed chelations. Loosening of a limited number of these does not necessarily lead to denaturation but does decrease the amount of thermal energy which must be absorbed to break away from the metastable native configuration. Once past the limit, the specific structure of the protein is lost and the peptide chain unfolds or rearranges to the denatured state. Reversibility of denaturation is assumed to be related to a sufficient retention of specific configuration to allow return to the fully native state to occur fairly readily under its guidance and in the proper environment.

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RAT GROWTH EXPERIMENTS WITH SOME DERIVATIVES OF PHENYLALANINE*

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Since the mechanism of the oxidation of phenylalanine to tyrosine is unknown, a more careful study of the chemical mechanism of the oxidation is desirable. The first possibility that might be considered is whether living tissue is able to attack directly and oxidize the carbon atom in the para position to the side chain of phenylalanine. In this case the 3-carbon amino acid side chain might be expected to be concerned only with the enzymatic specificity of the reaction and provide the point of attachment by which the enzyme or enzymes concerned are able to combine with the molecule before carrying out the oxidation at another point in the molecule. That such a direct oxidative attack on the aromatic nucleus is possible seems certain since benzene itself has been shown to be oxidized to phenol by animal tissues (1).

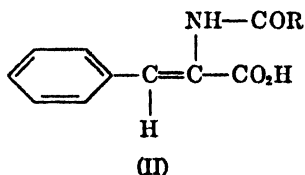
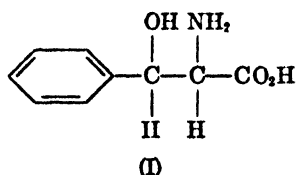
A second possibility involves the combination of phenylalanine with an enzyme, followed by an initial attack on the β -carbon of the side chain, an electronic rearrangement, an attack of the para position in the phenyl nucleus rather than the β position of the amino acid side chain, and subsequent reactions leading to tyrosine. That this position might be peculiarly susceptible to such an attack is indicated by previous work on the metabolism of alkyl-substituted benzenes, in which case hydrogen atoms on the α -carbon to the benzene nucleus appear to be more susceptible than those farther from the aromatic ring (1). The additional lability acquired by the simultaneous position as β to a carboxyl group should make this position quite sensitive to an oxidative attack.

The experiment reported in this paper was designed to test certain aspects of the second of these possibilities. An attempt was made to find suitable derivatives of phenylalanine which, *in vivo*, could be converted into tyrosine but not into phenylalanine. β -Phenylserine (I) and two stable *N*-acyl-substituted dehydrophenylalanines (II), all of which might be regarded as oxidized derivatives of phenylalanine and isomeric with tyrosine as far as the state of oxidation is concerned, were chosen as test compounds.

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The metabolic fate of β -phenylserine in animals was studied several years ago by both Dakin (2) and Knoop (3) in connection with their studies of the oxidation of ω -phenyl-substituted fatty acids and amino acids. They found β -phenylserine to be oxidized to form benzoic acid, which was excreted as hippuric acid. The yields of hippuric acid obtained in their experiments were low, however, and at that time there was no convenient way to detect whether the compound was partially transformed into either phenylalanine or tyrosine along with its oxidation to benzoic acid.

The development of the technique of measuring the rate of growth of young white rats receiving purified diets has now made it relatively simple to ascertain whether derivatives of the so called "essential" amino acids are able to serve as their precursors. This paper reports the diets used in investigating some derivatives of phenylalanine and tyrosine and the results obtained by administering β -phenylserine, acetyldehydrophenylalanine, and glycyldehydrophenylalanine to young white rats in such experiments.



EXPERIMENTAL

Litters of young white rats (Sprague-Dawley) were used for the feeding experiments. The basal synthetic diet had the following composition: amino acid mixture (exclusive of phenylalanine and tyrosine) 20.0, sucrose 43.0, hydrogenated vegetable oil 26.0, salt mixture (General Biochemicals, Inc., Salt Mixture 2) 4.0, cod liver oil 5.0, and agar 2.0 parts, respectively. The basal amino acid mixture was that of Rose and Rice (4), with minor alterations, and is reported in Table I. The water-soluble vitamins were given as a solution; each rat received daily 1 cc. of a solution containing 20 γ of thiamine hydrochloride, 20 γ of riboflavin, 20 γ of nicotinic acid, 20 γ of pyridoxine hydrochloride, 100 γ of calcium *d*-pantothenate, 25.0 mg. of choline chloride, and 20.0 mg. of sucrose (to make the solution palatable). The experimental compounds were incorporated in the diets in the amounts shown in Tables II and III and an equal weight of sucrose was omitted from the diet. Food and water were allowed *ad libitum*; the food consumption was measured daily and the animals were weighed at intervals of several days.

DL-Phenylalanine and L-tyrosine of satisfactory purity were purchased from commercial sources. β -Phenylserine (5), acetyldehydrophenylalanine (6), phenylpyruvic acid (7), glycyldehydrophenylalanine (8), and *N*-acetyl-DL-phenylalanine (9) were prepared as described in the literature. The purity of all these compounds was established by elementary analysis and by a comparison of their physical properties with those previously reported.

Diets were prepared in the following manner in order to detect the formation of either phenylalanine or tyrosine from the test compounds. To study phenylalanine production, a diet completely devoid of phenyl-

TABLE I
Amino Acid Mixture

	<i>gm. per kg. diet</i>
Glycine	6.0
DL-Alanine.	5.0
DL-Valine.	22.0
L-Leucine.	22.0
DL-Isoleucine.	18.0
L-Cystine.	2.0
DL-Methionine.	8.0
DL-Threonine.	14.0
DL-Aspartic acid.	10.0
L-Glutamic "	20.0
DL-Tryptophan	9.0
L-Arginine monohydrochloride.	9.0
L-Lysine monohydrochloride	25.0
L-Histidine monohydrochloride monohydrate.	10.0
Sodium bicarbonate.	20.0
Total	200.0

alanine was used. After an initial period of a few days, essentially the same daily loss in weight was noted for rats receiving either no supplement at all or a 1.5 per cent tyrosine supplement to the basal diet. An increase in growth rate, therefore, would indicate the formation of phenylalanine. For the study of tyrosine formation, a diet containing a 0.4 per cent phenylalanine supplement to the basal diet was found to maintain weight without gain or loss for a considerable period of time. As was reported by Womack and Rose (10), supplementation of this diet with either phenylalanine or tyrosine resulted in an increased rate of growth. If growth were obtained on this diet, a parallel experiment with a phenylalanine-deficient diet would reveal whether tyrosine or phenylalanine had been produced.

In the course of establishing these basal diets, it was noted that when diets having this basal composition were fed to rats weighing 50 to 100 gm. significantly better growth could be obtained at a level of 1.2 per cent DL-phenylalanine than when a 0.9 per cent supplement was used. This has been repeatedly noted with other animals and in other litters than the one reported here. This would appear contrary to the results reported by Rose and Womack (11), who found that a level of 0.9 per cent phenylalanine in the diet was optimum for growth. It should be emphasized, however, that our basal diet is slightly different in composition from theirs and our experiments were of limited duration and do not necessarily bear any relation to optimum conditions for growth over an extended period of time.

The results presented in Table II leave no doubt that both *N*-acetyldehydrophenylalanine and β -phenylserine have no significant growth-promoting ability for young rats under these conditions, and β -phenylserine actually appears to have a slight toxicity. The report by Beerstecher and Shive (12) that β -phenylserine inhibits the growth of various microorganisms, and that this inhibition may be removed by the addition of phenylalanine, is suggestive of the reason for the slight toxicity. The other stereoisomer of acetyldehydrophenylalanine and the other possible diastereoisomeric pair of β -phenylserines were not included in this experiment. Inasmuch as allothreonine can in no way serve as a substitute for threonine, it cannot be presupposed that the other form of β -phenylserine would not be available in lieu of either phenylalanine or tyrosine in the diet. A considerable amount of unsuccessful effort was expended in an attempt to prepare the other isomer; several reports of such an isomer occur in the literature, but attempts to produce the desired compound led either to β -phenylisoserine or to impure β -phenylserine of the same configuration as that used in this experiment.

A parallel experiment with *N*-acetyl-DL-phenylalanine showed that if acetyldehydrophenylalanine was reduced to acetylphenylalanine *in vivo* it would have resulted in good growth. *N*-Acetyl-DL-phenylalanine was fed at two levels in the diet, one in which the DL compound was equimolar with 1.2 per cent DL-phenylalanine and at a level double this amount. Animals receiving the larger amount (L component equimolar with 1.2 per cent phenylalanine) grew at the same rate as those receiving an equivalent amount of phenylalanine. Those receiving the lower amount either lost weight or failed to grow. This confirms the previous findings that *N*-acetyl-DL-phenylalanine is available as a precursor for phenylalanine (13) and that *N*-acetyl-D-phenylalanine cannot be used by the dog (9).

Glycyldehydrophenylalanine proved to be as readily available for

TABLE II
Phenylalanine-Deficient Diet

Rat No.	Initial weight	Days	Average daily food intake	Average daily weight change	Supplement to basal diet
	gm.		gm.	gm.	per cent
25 ♂	65	0-5	2.4	-2.0	None
		6-30	3.9	+1.1	0.9 DL-phenylalanine
		31-38	3.1	-0.6	2.4 acetyldehydrophenylalanine
26 ♂	70	0-5	2.6	-2.0	None
		6-15	2.0	-1.0	1.0 phenylserine
		16-30	2.5	-0.4	1.2 acetyldehydrophenylalanine
27 ♂	54	0-5	2.4	-1.6	None
		6-18	1.8	-1.1*	2.0 phenylserine
28 ♂	69	0-5	2.4	-1.8	None
		6-15	1.9	-1.4	2.0 phenylserine
		16-20	1.8	-1.2	2.0 " + 30 mg. phenylserine
					per day†
		21-30	2.0	0	2.4 acetyldehydrophenylalanine
29 ♂	76	0-5	2.8	-2.4	None
		6-30	2.4	-0.6	"
		31-45	2.3	-0.3	"
36 ♀	44	0-5	2.8	-1.80	"
		6-20	3.7	+0.80	0.9 DL-phenylalanine
		21-34	5.0	+1.64	1.2 "
37 ♂	57	0-5	3.0	-1.60	None
		6-17	4.3	+1.00	1.72 glycyldehydrophenylalanine
		18-20	2.7	-2.33	None
		21-30	5.1	+1.60	3.0 acetyl-DL-phenylalanine
		31-40	3.2	-0.70	1.5 "
38 ♀	53	0-5	3.0	-1.60	None
		6-20	4.4	+1.67	1.72 glycyldehydrophenylalanine
		21-24	2.3	-2.25	None
		25-30	3.7	0.0	1.5 acetyl-DL-phenylalanine
		31-40	4.4	+1.1	3.0 "
39 ♂	54	0-5	2.8	-1.80	None
		6-24	2.3	-0.33	1.5 L-tyrosine
40 ♂	66	0-5	3.0	-2.20	None
		6-24	2.6	-0.79	"
		25-34	4.8	+2.10	1.3 phenylpyruvic acid

* Animal in jeopardy of life.

† Approximately equal to and supplemental to the amount the animal received in the diet.

growth as phenylpyruvic acid or phenylalanine itself. This was to be expected since enzyme studies had shown it to be readily hydrolyzed *in vitro* to phenylpyruvic acid, glycine, and ammonia (14).

The above results on the availability of acetyl- and glycyldehydro-

TABLE III
Tyrosine-Deficient Diet

Rat No.	Initial weight	Days	Average daily food intake	Average daily weight change	Supplement to basal diet
	gm.		gm.	gm.	per cent
30 ♀	58	0-5	2.8	-0.6	None
		6-33	3.4	+0.9	0.6 L-tyrosine
31 ♀	73	0-5	3.4	-1.4	None
		6-15	2.7	-0.2	0.6 phenylserine
		16-40	4.0	+0.3	0.8 acetyldehydrophenylalanine
32 ♀	64	0-5	2.8	-1.0	None
		6-20	2.4	-0.5	1.2 phenylserine
33 ♀	62	0-5	2.8	-1.2	None
		6-20	2.7	-0.2	1.2 phenylserine
		21-27	2.4	-0.1	1.2 " + 25 mg. phenylserine per day*
34 ♀	59	0-5	2.6	-1.0	None
		6-15	2.5	0	"
		16-40	3.2	+0.1	1.2 acetyldehydrophenylalanine
35 ♀	65	0-5	2.6	-0.6	None
		6-15	2.4	0	"
		16-40	3.0	0	"
41 ♀	53	0-7	3.6	-0.43	"
		8-17	4.0	+1.16	0.6 L-tyrosine
		18-28	4.6	+1.27	0.6 "
42 ♂	53	0-7	3.6	-0.59	None
		8-17	3.1	+0.30	1.2 acetyldehydrophenylalanine
		18-30	3.2	+0.08	1.2 "
		31-37	3.6	+0.71	40 mg. tyrosine per day
43 ♀	58	0-7	3.6	-0.86	None
		8-17	3.2	0	2.4 acetyldehydrophenylalanine
		18-30	3.2	-0.08	2.4 "
		31-37	2.4	+0.14	50 mg. " per day†
44 ♂	60	0-7	4.1	-0.59	None
		8-17	3.6	+0.30	"
		18-30	3.7	+0.15	"
		31-37	3.0	+0.14	50 mg. acetyldehydrophenylalanine per day†
45 ♀	59	0-7	3.6	-0.86	None
		8-17	3.0	-0.10	"
		18-30	3.5	-0.15	"
		31-37	3.6	+0.16	"

* Approximately equal to and supplemental to the amount received in the diet.

† Dissolved in dilute NaOH, adjusted to pH 7, and given by intraperitoneal injection.

phenylalanine parallel the findings of Hoberman and Fruton, who used acetyldehydrotyrosine and acetyl-DL-tyrosine containing N¹⁵ (15). They

found that administration of N^{15} -labeled acetyldehydrotyrosine was not followed by the appearance of N^{15} in the excreted urea, whereas the administration of N^{15} -labeled acetyltyrosine resulted in a prompt appearance of N^{15} in the excreted urea.

The results presented in Table III are quite as definite in showing that β -phenylserine and acetyldehydrophenylalanine cannot give rise to tyrosine under these conditions.

SUMMARY

Synthetic diets are described with which it is possible to detect the formation of either phenylalanine or tyrosine from possible dietary precursors.

Glycyldehydrophenylalanine can be converted to phenylalanine by the growing white rat, presumably by hydrolysis to yield phenylpyruvic acid, which is then aminated to form phenylalanine.

The more readily prepared stereoisomers of acetyldehydrophenylalanine and β -phenylserine cannot be transformed into either phenylalanine or tyrosine under the same conditions.

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METABOLISM OF TESTOSTERONE AND 17-METHYL-TESTOSTERONE BY LIVER TISSUE*

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Both 17-methyltestosterone and testosterone have been shown to be inactivated by the liver *in vivo* (1-3). The metabolism of the two compounds, however, does not follow the same course. The evidence that the two steroids differ in their metabolism *in vivo* is 3-fold: (a) the ratio of the androgenic activity of methyltestosterone given orally to that given subcutaneously is greater than the same ratio for testosterone (4); (b) when testosterone is administered, there is an increase in 17-ketosteroids in the urine, while when methyltestosterone is given, this level remains constant or decreases (5-10); and (c) when methyltestosterone is administered, blood and urine levels of guanidoacetic acid and creatine are elevated (11-13), while when testosterone is given, the levels are decreased (13).

The present experiments were undertaken to see whether any differences observed in the *in vitro* metabolism of the two steroids by liver tissue could explain the observed differences in their *in vivo* metabolism. The results presented in this paper offer a possible explanation for the differences noted in (a) and (b) above, but not for the effects on creatine metabolism.

EXPERIMENTAL

Tissues of the following animals were used in these studies: chicken, rat, rabbit, guinea pig, dog, monkey, and human.

For all species except the human, the tissue was removed at the site of the experiment. The dog and monkey were sacrificed by fatal doses of anesthetic and the smaller species were sacrificed by decapitation. In the case of the human, tissue was removed in the operating room and

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immediately placed in ice-cold Locke's solution. It was then rushed to the laboratory, sliced, and incubated with minimal delay.¹

The handling of the animals, the preparation of the minces used, and the analytical procedures involved have been described by Samuels, McCauley, and Sellers (14). The liver tissue was cooled in an ice bath immediately after removal from the animal. It was then minced and aliquots were weighed and washed into incubation flasks with 25 cc. of a modified Krebs phosphate buffer solution, pH 7.4. Cofactors, when used, were added to the buffer solution. Incubation was in oxygen for 1 hour at 37.5°.

Results

Liver tissue of all of the species studied contained enzymes which acted on the α,β -conjugated unsaturated ketone structure in ring A of both testosterone and methyltestosterone (Table I). Within the limits of experimental error the action in this ring in the two compounds was similar in all species.

The action on carbon-17, however, differed markedly. While the livers of all of the species tried were able to synthesize 17-ketosteroids from testosterone, none of them produced such compounds from methyltestosterone. There was no evidence of oxidative splitting off of the methyl group.

The effect of added diphosphopyridine nucleotide (DPN) on the metabolism of testosterone by liver tissue of the dog, chicken, rat, guinea pig, monkey, and human differed from that on methyltestosterone. The formation of 17-ketones was markedly increased with the former steroid, while there was still complete failure to obtain any such compound from the latter. At the same time the nucleotide increased the rate of destruction of the α,β -ketone conjugation in testosterone but had little effect on the rate of destruction of this group in methyltestosterone. This observation would add support to the concept, originally based on sequential studies with chicken and rat livers (15), that in addition to an enzyme acting on ring A of testosterone there is another system affecting the conjugated double bond structure in this ring which acts preferentially on Δ^4 -androstenedione-3,17.

The rabbit liver differed from the others. The rates of destruction of the α,β -conjugated ketone groups of the two steroids were similar in the presence of tissue alone and were not accelerated in either case by the addition of DPN. Also, as observed before (15), the nucleotide did not increase the formation of 17-ketosteroids from testosterone.

¹ We wish to thank Dr. N. F. Hicken and Dr. R. G. Weaver for obtaining the human tissues for us.

TABLE I

Metabolism of Testosterone (T) and Methyltestosterone (MT) by Minced Liver Tissue of Various Species; Influence of DPN and Citrate

The results are expressed as micromoles of steroid per gm. of tissue per hour.

Species	Steroid	Buffer only		Buffer + DPN		Buffer + citrate	
		α,β destruction	17-Ketosteroid formed	α,β destruction	17-Ketosteroid formed	α,β destruction	17-Ketosteroid formed
Rat	T	0.68	0.25	0.91	0.63	1.10	0.15
		0.62-0.75	0.19-0.31	0.86-0.94	0.50-0.76	1.03-1.22	0.11-0.18
"	MT	0.68	0	0.65	0	1.07	0
		0.56-0.82		0.42-0.97		0.88-1.22	
Guinea pig	T	0.33	0.36	0.58	0.47	0.54	0.09
		0.18-0.51	0.33-0.38	0.52-0.62	0.36-0.62	0.53-0.56	0.08-0.11
" "	MT	0.24	0	0.31	0	0.37	0
		0.18-0.30		0.31-0.32		0.32-0.40	
Dog	T	0.32	0.11	0.54	0.35	0.41	0.11
		0.27-0.36	0.10-0.13	0.48-0.57	0.34-0.36	0.36-0.44	0.10-0.12
"	MT	0.30	0	0.35	0	0.34	0
		0.23-0.36		0.33-0.38		0.32-0.36	
Monkey	T	0.54	0.46	0.73	0.59	0.99	0.43
		0.51-0.57	0.42-0.52	0.71-0.77	0.51-0.66	0.99-1.00	0.41-0.45
"	MT	0.65	0	0.82	0	1.02	0
		0.60-0.70		0.80-0.84		0.97-1.07	
Human (A. H.)	T	0.77	0.45			1.01	0.33
		0.77, 0.78	0.41, 0.50			0.96, 1.06	0.30, 0.36
Human (A. H.)	MT	0.63	0	0.54	0	1.18	0
		0.58-0.78		0.49-0.59			
Human (L. R.)	T	1.06	0.28	1.62	0.64		
		1.02, 1.10	0.23, 0.33	1.34, 1.90	0.63, 0.65		
Rabbit	T	0.63	0.33	0.54	0.34	0.88	0.12
		0.59-0.67	0.30-0.37	0.52-0.58	0.32-0.35	0.86-0.90	0.11-0.15
"	MT	0.48	0	0.48	0	0.78	0
		0.42-0.55		0.33-0.56		0.76-0.80	
Cockerel	T	1.36	0.66	1.81	1.17	2.19	0.47
		1.33-1.41	0.57-0.84	1.69-2.04	1.08-1.24	1.98-2.32	0.37-0.58
"	MT	1.20	0	1.40	0	1.34	0
		1.10, 1.30		1.20-1.60		0.98-1.52	

The figures on the second lines represent the range of values obtained for individual incubation flasks. In most instances, three flasks were incubated; in a few, only two, indicated by a comma. Tissues from the same animals were used for both testosterone and methyltestosterone. Each flask contained approximately 0.5 gm. of liver mince, 2.00 μ M of steroid, and 25 cc. of a buffer solution containing the following: NaCl 0.08 M, KCl 0.0056 M, MgCl₂ 0.0021 M, Na₂HPO₄-NaH₂PO₄ buffer (pH 7.4) 0.04 M. The flasks were incubated in O₂ for 1 hour at 38°.

When citrate was added to the incubation medium, the destruction of the conjugated system giving the 240 m μ absorption band was in-

creased in all cases except in the dog (Table I). This lack of influence of citrate in the presence of dog tissues has also been observed when kidney slices were incubated with testosterone (16). The increase was of the same order for both steroids.

DISCUSSION

Sweat, Samuels, and Lumry (17) have been able to prepare and characterize an enzyme system from liver that catalyzes the reaction, testosterone + DPN \rightarrow Δ^4 -androstenedione-3,17 + DPNH₂. This reaction is apparently the one which proceeds in the liver when an increased response to the Zimmermann reaction is found after incubation. As has been shown in this paper, if methyltestosterone is incubated under the same conditions as those used for testosterone, there are no compounds formed that give the Zimmermann reaction. It would appear that the enzyme system involved is able to oxidize a secondary but not a tertiary alcohol in this position; the methyl group therefore remains attached. This failure to demethylate the steroid then prevents the action of the enzyme requiring a 17-ketone group which acts on the conjugated system in ring A.

Apparently the presence of a methyl group on carbon-17 does not interfere with either formation or breakdown of the enzyme-substrate complex involved in the reaction on ring A catalyzed by citrate since, within the limits of the method, the effect of the cofactor on the rate of reaction was the same with both steroids.

These results offer a rational explanation of at least two of the differences observed in the over-all effects of the two steroids. The lack of any marked demethylation of methyltestosterone in the liver would prevent the comparative decrease in androgenic activity which occurs when testosterone is converted to Δ^4 -androstenedione-3,17 and would also, by elimination of the action of the additional enzyme system, lower the rate at which the conjugated structure in ring A would be lost. It would also explain the failure to observe an increase in excretion of 17-ketosteroids after administration of methyltestosterone. One might expect, however, that methylated steroids such as the methylandrostanediols might be found in the urine or bile.

While the results of this investigation are consonant with two of the differences in testosterone and methyltestosterone metabolism, they do not explain the influence of the latter on creatine formation. The lack of evidence for demethylation makes highly improbable the rôle of the steroid as a methyl transfer agent. The results conform with the concept that the influence of the steroid is on the formation of guanidoacetic acid (13), but give no affirmative evidence.

SUMMARY

The conjugated system in ring A which gives maximal absorption at 240 m μ was destroyed in both testosterone and 17-methyltestosterone by liver tissue of dog, rat, chicken, guinea pig, monkey, rabbit, and human.

Substances giving the Zimmermann reaction for 17-ketosteroids were always formed in the case of testosterone but not in the case of methyltestosterone.

When DPN was added to the incubation mixture the formation of 17-ketosteroids from testosterone and the destruction of the α,β -ketone structure were both increased in all species but the rabbit. The nucleotide, however, had no effect on methyltestosterone. The failure of the nucleotide to influence the metabolism of ring A in the latter steroid would indicate that the methyl group on carbon-17 prevents the action of an additional enzyme acting on ring A.

When citrate was added as cofactor the destruction of the conjugated double bond system in ring A increased similarly in both steroids and in all species except the dog. There was no increase in formation of 17-ketosteroids. The presence of a methyl group on carbon-17 apparently does not block the action of this enzyme.

These observations would explain some of the differences in the metabolism of the two androgens.

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POLYPEPTIN,* AN ANTIBIOTIC FROM A MEMBER OF THE *BACILLUS CIRCULANS* GROUP

II. PURIFICATION, CRYSTALLIZATION, AND PROPERTIES OF POLYPEPTIN†

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Polypeptin is an antibiotic which has been isolated from the culture fluid of *Bacillus krzemieniewski*, a mucoid variant of *Bacillus circulans*. It inhibits the growth of bacteria and fungi, hemolyzes erythrocytes, and is toxic to mice. A description of the organism and the bacteriological aspects of polypeptin have been reported (2).

Polypeptin is prepared from the 10 day-old fermentation liquid obtained by growing the *B. krzemieniewski* in alanine medium (2). The 10 day culture fluid contains 100 units¹ of polypeptin per ml. and has a pH value of 6.7 to 7.0.

Purification of Polypeptin—To 30 liters of the culture fluid, adjusted to pH 2.0 by adding 200 ml. of concentrated hydrochloric acid, 10 liters of acetone are added and the mixture is mechanically stirred for 10 minutes. 23 kilos of ammonium sulfate are added, the stirring is resumed for a further 10 minutes, and the mixture allowed to stand for 5 minutes. The bottom layer is then discarded. The upper layer is freed of precipitated protein by suction through cheese-cloth. The precipitate is washed with 2 liters of acetone and then discarded. The combined acetone extracts are concentrated to one-half volume by distillation *in vacuo*

* This antibiotic was named "circulin" in the previous reports from the Venereal Disease Research Laboratory (1-3). In an agreement with Professor Tetrault, the name of the antibiotic isolated from a strain of *Bacillus circulans* in the Venereal Disease Research Laboratory has been changed to "polypeptin," whereas the antibiotic isolated by the group from Purdue University will continue to be known as "circulin."

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¹ The antibiotic assays were carried out by Dr. C. McLeod of the Bacteriology Section, Venereal Disease Research Laboratory, United States Public Health Service (2). An 18 hour serial dilution assay was used with *Escherichia coli* as the test organism.

at 50°. The concentrated extract is shaken with 12 liters of chloroform, when the polypeptin precipitates as a waxy substance. The upper layer is washed twice with smaller volumes of chloroform and filtered through paper. The waxy solid is dissolved in 600 ml. of warm 80 per cent acetone and the filtered solution, which contains over 50 per cent of the antibiotic activity found in the original culture fluid, is cooled at -20° for 3 days. The precipitate is collected by filtration at -20° and dissolved in 500 ml. of warm 65 per cent ethyl alcohol; the solution is cooled gradually and finally maintained at -20° for several days. Microscopic examination of the resulting precipitate may reveal triangular prisms; complete crystallization may be attained by allowing the preparation to stand at room temperature. The crystallization of polypeptin is relatively slow but large (5 mm.) triangular prisms may be obtained by repeated cooling and warming to room temperature. The crystals are easily broken by manipulation of suspensions.

Dry preparations of crystalline polypeptin may be obtained without loss of hemolytic and antibiotic activity. Solutions of the crystals in 65 per cent ethyl alcohol are evaporated to dryness by vacuum distillation at 37°. The tan-colored crystalline residue is then dried *in vacuo* over phosphorus pentoxide at room temperature. The over-all yield from 30 liters of culture fluid (originally containing 3,000,000 units) averages 7.5 gm. or 1,500,000 *Escherichia coli* dilution units (Table I, Preparation A).

In order to recrystallize polypeptin, the dry crystalline polypeptin is dissolved in a minimum quantity of warm 65 per cent ethyl alcohol; the resulting saturated solution is cooled gradually to -20° and maintained at this temperature overnight. Polypeptin separates as triangular prisms (Fig. 1, A) which yield light tan-colored solutions in water. These aqueous solutions have a pH value of 5.5 and retain both antibiotic and hemolytic activities (Table I, Preparation B).

*Analysis*²—C 47.0, H 8.0, N 12.35, S 3.6, ash 0.07

Apparent mol. wt. (cryoscopic method in phenol) 1050

Chemical Character of Polypeptin—Qualitative tests for alkali-labile sulfur (4) and for sulfhydryl groups (5) in polypeptin were negative. When polypeptin was hydrolyzed and tested by the method of Mease (6), the value for hydrolyzable sulfate sulfur was the same as that for total sulfur. The sulfur in polypeptin therefore occurs as sulfate or in a form easily converted to sulfate (7).

The sulfur content as determined by the method of Pollock and Partansky (8) is unaltered on recrystallization from water with the aid of chloroform, but when polypeptin was dissolved in 2.5 M sodium acetate

² Elek Micro Analytical Laboratories, Los Angeles, California.

solution at pH 8.3 and reprecipitated with chloroform, the sulfur content was greatly reduced (Table II). Polypeptin prepared by precipitation

TABLE I

Antibiotic and Hemolytic Activities of Purified Polypeptin Preparations

All of the preparations had identical hemolytic activities of 8000 dilution units.

Preparation	Method of purification	Antibiotic activity
		units per mg.
A	Crystallized from ethyl alcohol	180
B	Twice recrystallized from ethyl alcohol	200
C	“ “ “ “ “ recrystallized from methyl alcohol	200
D	Crystallized from ethyl alcohol; recrystallized from methyl alcohol	240
E	Twice recrystallized from ethyl alcohol; recrystallized from isopropyl alcohol	160
	Twice recrystallized from ethyl alcohol; ether-crystallized	180
G	Twice recrystallized from 1.0 M NaAc	180
H	Polypeptin acetate	160
I	NH ₄ OH precipitation	200
J	NaOH precipitation neutralized with HCl	200
K	Sulfuric acid-soluble; precipitated by ammonium sulfate	180
	Phenol-soluble; ether-precipitated	200

TABLE II

Sulfur Content of Polypeptin Purified by Four Procedures

Batch No.	Twice recrystallized from 65 per cent ethyl alcohol, pH 5.5			Precipitated by CHCl ₃ , pH 5.5		Precipitated by sodium acetate, pH 8.3		Precipitated at pH 11.0	
	Total sulfur	Hydrolyzable sulfate as sulfur	Ash	Total sulfur	Ash	Total sulfur	Ash	Total sulfur	Ash
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
22	3.94	3.84	0.05	3.91	0.14	0.41	4.47	0.18 by NaOH	1.7
20	3.80	3.94	0.14			0.60	3.91	0.18	1.2
60	3.61	3.68	0.14	3.9	0.06	0.43	2.76	0.24 by NH ₄ OH	0.07
63	3.91	3.78	0.16	3.85	0.06	0.34	2.82	0.35	0.06

with sodium or ammonium hydroxide at pH 11.0 was found to contain only a trace of sulfur (Table II). Evidently the crystalline polypeptin is a sulfate of a base.

The biuret (4) and ninhydrin (4) tests are the only color tests which

have given significantly positive responses. The Schoenheimer-Sperry test (9) for cholesterol produced a yellow color upon standing for 10 minutes. In recrystallized preparations less than 0.05 per cent of tryptophan can be detected (10).

The Van Slyke amino nitrogen content of polypeptin is 3.6 per cent. The determination of ninhydrin-carbon dioxide in an acid hydrolysate of polypeptin shows the presence of 5.7 per cent ninhydrin-carbon. Paper chromatography of the hydrolysate indicates the presence of an amino acid comparable to the α, γ -diaminobutyric acid found in polymyxins (11) and other amino acid spots.^{3, 4} Polypeptin sulfate is not inactivated at room temperature by pepsin at pH 2.0, trypsin at pH 8.0, hydrogen sulfide, or ferric chloride. Dilute solutions of iodine in potassium iodide precipitate polypeptin from water solution. When the iodine is reduced with an equivalent quantity of thiosulfate, the polypeptin redissolves, retaining its antibiotic and hemolytic activities. Polypeptin sulfate dialyzes rapidly through viscose membranes.

Concentrated sulfuric acid dissolves 6.0 per cent of its weight of dry, twice recrystallized polypeptin sulfate at 20° without charring. Most of the polypeptin can be recovered by diluting with ammonium sulfate solution, neutralizing with ammonium hydroxide, and extracting with acetone. A different type of crystal, parallelepiped in shape, was obtained when this product was crystallized from 65 per cent ethyl alcohol. The antibiotic and hemolytic activities were unaltered (Table I, Preparation K).

In addition to the triangular prisms obtained when polypeptin sulfate is crystallized from 65 per cent ethyl alcohol-water solutions, a number of other crystalline forms are obtained by recrystallization from other solvents. The preparation used in the recrystallization procedures described below was recrystallized twice from 65 per cent ethyl alcohol, freed of alcohol *in vacuo* at 37°, and then dried to constant weight over phosphorus pentoxide *in vacuo* at room temperature.

When a solution containing 0.25 per cent of polypeptin sulfate in warm 30 per cent ethyl alcohol was cooled overnight at 0°, hexagonal plates formed.

A 4 per cent solution of polypeptin sulfate was prepared by adding the dry powder to absolute methyl alcohol at room temperature. Crystallization began after a few minutes at room temperature and, after a few hours, over 90 per cent of the polypeptin sulfate was recovered as crystal-

³ The author is indebted to Dr. J. R. Weisiger and Dr. W. Hausmann of The Rockefeller Institute for Medical Research for these determinations.

⁴ The investigation of the purity of polypeptin is being continued by Dr. L. C. Craig at The Rockefeller Institute for Medical Research by the counter-current distribution procedure. A preliminary forty-five tube transfer indicates that polypeptin is 90 per cent one component.

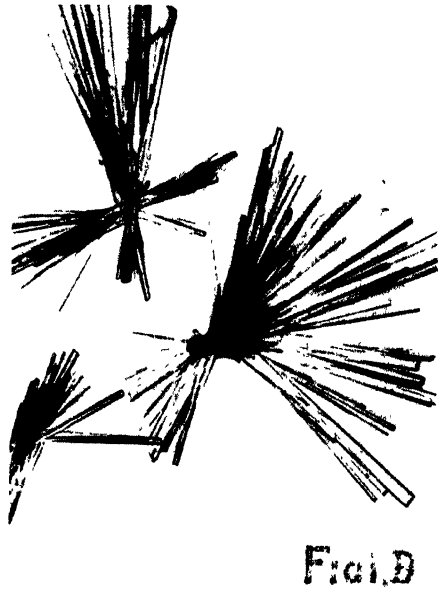
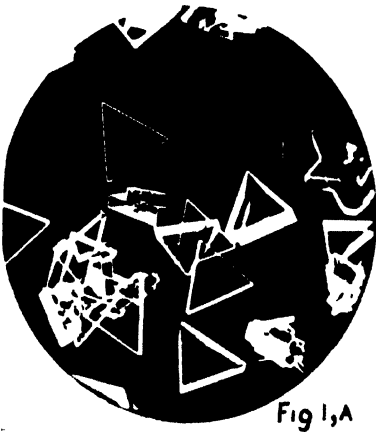


FIG 1. Polypeptin sulfate recrystallized from (A) 65 per cent ethyl alcohol, (B) absolute methyl alcohol, and (C) 70 per cent isopropyl alcohol, and (D) polypeptin recrystallized from 1 M sodium acetate solution. Magnification 160 \times . Photography by Dr Robert D. Wright and John Louis Bruns.

line material (Fig. 1, *B*; Table I, Preparation C). With less pure samples, several days incubation at room temperature may be required for crystals to appear (Table I, Preparation D).

A 6 per cent solution of polypeptin sulfate in warm 70 per cent isopropyl alcohol yielded crystals when cooled at 2° (Fig. 1, *C*; Table I, Preparation E).

A 1 per cent solution of polypeptin sulfate in warm 80 per cent acetone was heated in water without stirring. The solution became turbid and rosettes of flat plates formed.

To a 0.3 per cent solution of polypeptin sulfate in cold water at pH 5.5, ethyl ether or chloroform was added to 5 per cent concentration. A crystalline precipitate formed, consisting of double pyramids or hexagonal plates with the respective solvent. Excess ether or chloroform should be avoided, since the precipitate may acquire an oily consistency (Table I, Preparation F).

There is no apparent difference in the properties or biological activity of the several crystalline forms of polypeptin sulfate. Any of these crystalline forms of polypeptin can be converted into triangular prisms by recrystallization from 65 per cent ethyl alcohol. Crystals of polypeptin sulfate have been obtained with 30 and with 65 per cent ethyl alcohol but not with other ethyl alcohol-water ratios or with absolute ethyl alcohol. Preparations which yield colorless solutions have been prepared by recrystallizing from 65 per cent ethyl alcohol, then from methyl alcohol, then from 65 per cent ethyl alcohol by the addition of absolute methyl alcohol, and finally from water solution with chloroform.

The polypeptin sulfate employed for the determination of the apparent molecular weight in phenol, recovered by addition of ether and recrystallization from 65 per cent ethyl alcohol, exhibited unchanged crystalline form, absorption spectrum, and biological activity (Table I, Preparation L).

The form in which polypeptin sulfate crystallizes is apparently dependent upon the solvent from which it is crystallized. This phenomenon is apparently similar to that found with catalase (12), lactoglobulin (13), ribonuclease (14), and lysozyme (15).

Solubility of Polypeptin Sulfate—Dry, twice recrystallized polypeptin sulfate is soluble at 25° in the following organic solvents: ethylene glycol (5 per cent), pyridine (8 per cent), glacial acetic acid (10 per cent), allyl alcohol (4 per cent), and phenol (12 per cent). It dissolves readily at 25° in absolute methyl alcohol (4 per cent) or ethyl alcohol (5 per cent), and after a brief period separates as an insoluble substance. It is insoluble in anhydrous acetone, ethyl ether, chloroform, amyl acetate, benzene, carbon disulfide, and petroleum ether.

The solubility of polypeptin sulfate in lower alcohols and ketones containing water increases with rise in temperature. The solubility in mixtures of water and ethyl alcohol at 25° was investigated (Fig. 2). An excess of dry, twice recrystallized polypeptin sulfate was added to a series of tubes containing 10 ml. of water-ethyl alcohol mixtures of concentrations ranging from 0 to 100 per cent. The tubes were stirred intermittently for 15 hours and centrifuged. Aliquots of the clear supernatant fluids were dried *in vacuo* to constant weight. Maximum solubility was obtained with 65 per cent ethyl alcohol. Although polypeptin sulfate dissolves readily in absolute ethyl alcohol, most of it precipitates as an

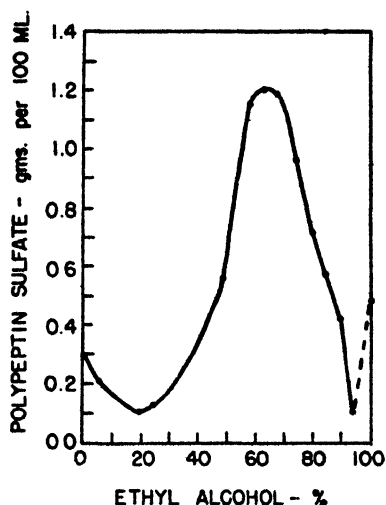


Fig. 2. Solubility of polypeptin sulfate in water-ethyl alcohol solutions at 25°

amorphous solid after 30 minutes at room temperature. The value shown for 100 per cent ethyl alcohol is that obtained after equilibrium is reached.

Recrystallized polypeptin sulfate dissolves slowly in water: 0.3 per cent at 25° and 0.4 per cent at 2°.

Polypeptin sulfate was found to be more soluble in acetate solutions than in water. The increase in solubility is probably due to the displacement of most of the sulfate anion by acetate ion. With ammonium acetate; maximum solubility (2 per cent) is obtained in 0.7 M solution at 25°. The pH of this solution is 7.0. With sodium acetate at 25°, a maximum solubility of 1 per cent is obtained in 0.7 M solution at pH 7.3. Polypeptin sulfate is less soluble in citrate or phosphate solutions than in acetate solution.

A 1 per cent solution of recrystallized polypeptin sulfate was prepared

in 1.0 M sodium acetate at 2° (pH 7.3). The solution was centrifuged to remove any insoluble material and then warmed to 25°. The solution became turbid on standing and long pointed needles separated (Fig. 1, D). Antibiotic and hemolytic activities were unchanged (Table I, Preparation G).

A polypeptin preparation which is very soluble in water can be prepared by precipitating polypeptin from a concentrated solution of sodium acetate. To a 1 per cent solution of recrystallized polypeptin sulfate in cold 0.7 M sodium acetate sufficient solid acetate was added to make the solution 2.5 M and the mixture was warmed to 25° (pH 8.3). The precipitate was collected by centrifugation, dissolved in a minimum amount of water, and the polypeptin precipitated with chloroform. Upon centrifugation, the precipitate collected as a gel at the interface. The solvents were decanted and the gelatinous precipitate was dissolved in water. Attempts were made to remove the sodium acetate by a second treatment with chloroform or ether, but the polypeptin was not reprecipitated. The product was neutralized with acetic acid and dried. Both antibiotic and hemolytic activities were retained (Table I, Preparation H). The dry preparation, which was very soluble in water and in 65 per cent ethyl alcohol, contained approximately 0.5 per cent sulfur and 3.5 per cent ash (Table II).

The solubility of polypeptin sulfate in citrate solutions varies with the concentration, temperature, and pH. At pH 7.0 the solubility of polypeptin in citrate buffer at 25° was found to be at a minimum (0.05 per cent) at 0.005 M. The same buffer readily dissolved 0.1 per cent of its weight of polypeptin at 2.0°; when the solution was warmed to 25°, it became turbid.

In order to determine the effect of pH upon the solubility, 0.01 M citrate or citric acid solutions were added to equal volumes of a 0.2 per cent aqueous solution of polypeptin sulfate at 25°. From pH 2.8 to 6.0 the polypeptin remained in solution. At pH 6.2 a turbidity appeared which was intensified as the pH was raised; at pH 7.0 flocculation occurred. Above pH 7.0 there was no change until the pH reached 9.5, when there was a definite increase in turbidity and flocculation. The effect of pH upon the solubility of polypeptin sulfate in phosphate and acetate buffers was the same as in citrate buffer. A more concentrated solution of polypeptin was required when acetate buffer was used.

Solubility Behavior in Alkaline Solution—The determination of the pH of minimum solubility was carried out at room temperature with a 0.1 per cent water solution of crystalline polypeptin sulfate at pH 5.0. Ammonium hydroxide or sodium hydroxide solutions of varying concentrations were used to adjust the polypeptin solutions to the desired pH value. 20 ml. quantities of the polypeptin solution in separate tubes

were adjusted to pH values over the range of 6.0 to 12.0 with ammonium hydroxide, and the solutions were then diluted with water to a total of 25 ml. The first faint turbidity occurred at pH 9.5 and increased to 11.0, at which maximum turbidity and flocculation occurred. There was no turbidity above pH 11.8. These precipitates dissolved when cooled to 2° and reappeared when warmed to 25°. The turbidity in the more alkaline tubes decreased gradually upon standing at room temperature. The minimum solubility point is at pH 11.0. At pH 11.5 the disappearance of the turbidity in 48 hours coincided with the inactivation of the polypeptin as shown below.

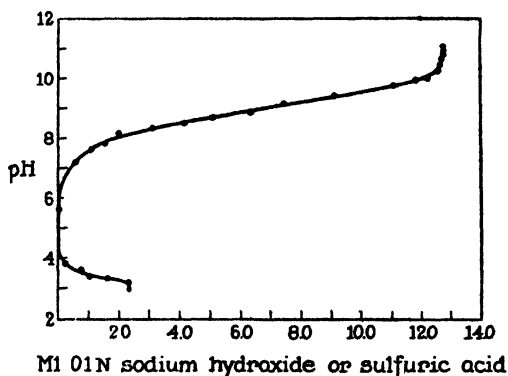


FIG. 3. Electrometric titration of 0.5 gm. of recrystallized polypeptin sulfate dissolved in 500 ml. of water at 0° (pH 5.6) with 0.1 N sodium hydroxide and 0.1 N sulfuric acid. The values obtained by titrating 500 ml. of water were subtracted from the values obtained by titrating the polypeptin sulfate solution. The resulting data, or the quantities of acid or alkali bound by the polypeptin sulfate, were used to plot the curve.

A water solution of recrystallized polypeptin sulfate of pH 5.6 was titrated electrometrically with 0.1 N sodium hydroxide and 0.1 N sulfuric acid at 0°. The Beckman pH meter equipped with a type E glass electrode was used to determine the pH of the solutions. The values obtained were corrected by subtracting the titration values obtained with an equal volume of water. The curve in Fig. 3 shows the quantities of acid and alkali which react with 0.5 gm. of polypeptin sulfate dissolved in 500 ml. of cold water. It shows that 1.27 m.eq. of OH^- ions and 0.23 m.eq. of H^+ ion are bound by 500 ml. of the polypeptin sulfate, indicating five or six basic (presumably amino) groups to one carboxyl, an equivalent weight of 2100 to 2200, and an amino nitrogen content of 3.56 per cent, in good agreement with the value obtained in the Van Slyke determination.

Preparation of Free Base—3 liters of 0.3 per cent solution of crystalline

polypeptin sulfate in water at pH 5.5 were adjusted at 2° to pH 11.0 with approximately 12 ml. of 26 per cent ammonium hydroxide. The heavy flocculent precipitate was immediately collected by centrifugation and washed with 50 ml. of water containing sufficient ammonium hydroxide to maintain the pH at 11.0. The precipitate was suspended in water, the ammonia and water were removed by vacuum distillation at 40°, and the free base was dried *in vacuo* over phosphorus pentoxide. 65 per cent of the polypeptin was recovered. Antibiotic and hemolytic activities were unchanged (Table I, Preparation I). The polypeptin contains no ash and only traces of sulfur and ammonia (Table II); in the dry state it is stable for at least 2 months (Table I, Preparation I). It is slowly soluble in water, yielding an 0.1 per cent solution at 2°. The pH of the solution is 9.8. The solubility of the free base in absolute methyl and

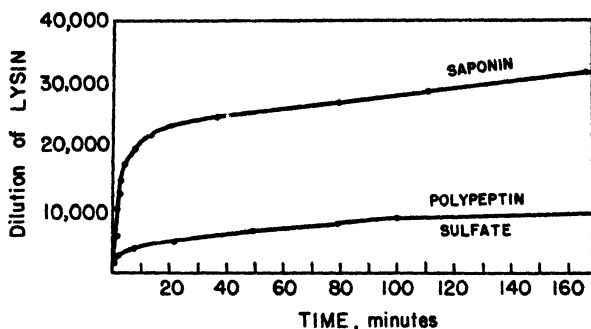


FIG. 4. Time-dilution hemolysis curves for polypeptin sulfate and saponin and human erythrocytes at 25°.

ethyl alcohols is very high. However, in methyl alcohol the free base soon precipitates.

Salts of polypeptin can be prepared by adding acids to the free base. Polypeptin sulfate so prepared (pH 5.0) crystallized from 65 per cent ethyl alcohol as triangular prisms. The chloride is described in Table I (Preparation J).

Comparison of Hemolytic Activity of Polypeptin Sulfate and Saponin—The hemolytic activity of twice recrystallized polypeptin sulfate has been determined by the time-dilution method of Ponder (16). Human erythrocytes in 1 per cent sodium chloride solution at pH 6.3 and at 25° were used. Solutions of saponin (pure, Merck) were used for comparison. According to this method saponin proved to be much more active than polypeptin. The times for complete hemolysis were very short in the more concentrated solutions of both lysins and, after a certain dilution was reached, complete hemolysis was greatly delayed (Fig. 4).

Physical Properties of Polypeptin Sulfate—Twice recrystallized polypeptin sulfate melts with decomposition in both the Thiele and the Fisher-Johns apparatus at 235°, recrystallized polypeptin acetate at 160°, and free base at 176°.

A 3 per cent solution of twice recrystallized polypeptin sulfate in 70 per cent isopropyl alcohol showed specific rotation $[\alpha]_D^{20} = -93.3^\circ$. The same value was observed with more dilute solutions in 65 per cent ethyl alcohol, 80 per cent acetone, 0.5 N sodium acetate, and 0.1 N HCl.

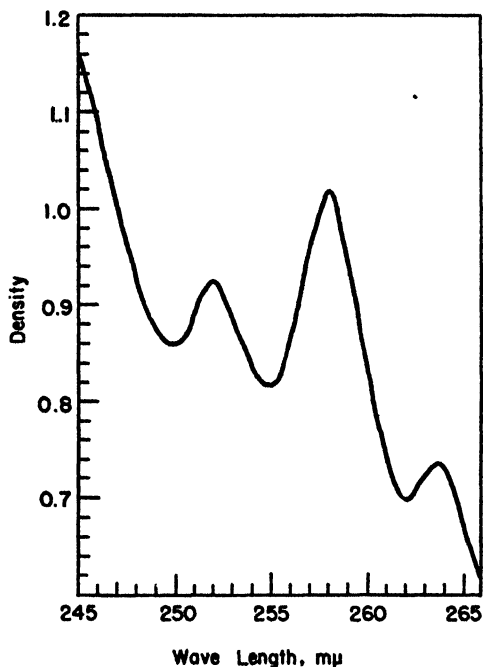


FIG. 5. Ultraviolet absorption spectrum of 0.7 per cent polypeptin sulfate in 70 per cent ethyl alcohol.

A 0.7 per cent solution of twice recrystallized polypeptin sulfate in 65 per cent ethyl alcohol, examined in the Beckman model DU photoelectric quartz spectrophotometer, showed complete absorption between 2000 and 2400 Å, three maxima at 2520, 2580, and 2640 Å, and almost complete transmission above 3000 Å (Fig. 5). These absorption bands indicate the presence of an unknown chromophore group or groups in polypeptin. Solutions of polypeptin in 0.5 N sodium acetate or 0.1 N HCl showed the same maxima.

Stability of Polypeptin Preparations—The dry sulfate can be stored *in vacuo* over phosphorus pentoxide or in a bottle under atmospheric

conditions for at least 1 year without decrease in activity. Non-sterile, 0.2 per cent solution of polypeptin sulfate in water at pH 5.0 and 0.7 per cent solution in 65 per cent alcohol or 80 per cent acetone retain their activities for at least 6 months at room temperature.

The free base stored *in vacuo* over phosphorus pentoxide retains its activity for at least 2 months.

Dry preparations of the free base and of the sulfate were heated in test-tubes in an oil bath for 5 minutes at various temperatures. For the determination of antibiotic and hemolytic activities the heated prepara-

TABLE III
Simultaneous Destruction of Both Antibiotic and Hemolytic Activities of Dry Polypeptin Preparations by Heat

Temperature	Antibiotic activity	Hemolytic activity, times diluted
Polypeptin, free base		
°C.	units per mg.	
Unheated	160	8000
160	160	8000
165	40	2000
170	20	1000
175 (m.p.)	10	<500
180	<10	<500
Polypeptin sulfate		
Unheated	160	8000
200	160	8000
220	80	4000
230	80	2000
235 (m.p.)	10	500
242	<10	<500

tions were dissolved in water and 0.9 per cent saline-phosphate solution, respectively. The antibiotic and hemolytic activities were not affected at temperatures below the melting point (Table III). Those preparations heated at temperatures which approximated their respective melting points lost both antibiotic and hemolytic activities simultaneously.

The effect produced upon the activity of polypeptin by incubating solutions of polypeptin at various pH values was investigated. Non-sterilized, 0.2 per cent water solutions of polypeptin sulfate (400 units per ml.), which had been recrystallized from methyl alcohol, were adjusted to various pH values from 2.2 to 11.0 with *N* HCl acid or *N* NaOH, diluted to standard volume, and stored at room temperature in glass-stoppered vessels. Aliquots of these solutions were removed at intervals

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and adjusted to pH 7.0 with a trace of phosphate buffer and 1.0 N hydrochloric acid or sodium hydroxide. Sodium chloride was also added to a part of each sample so that it would contain the equivalent of 0.9 per cent sodium chloride for the hemolysis test.

The hemolytic activity was determined as follows: Each 1.0 ml. of sample in 0.9 per cent saline-phosphate, pH 7.0, was serially diluted with the saline-phosphate buffer, and 1.0 ml. of a 0.75 per cent suspension of washed and packed human erythrocytes was added to each tube. The tubes were incubated at 25°. The maximum dilution of the dry preparation producing complete hemolysis at the end of 1 hour was used as the hemolytic activity.

The data in Table IV show that both antibiotic and hemolytic activities of polypeptin are retained at pH 2.5 and 5.5 for 29 days. Poly-

TABLE IV
Effect of pH upon Antibiotic and Hemolytic Activities of Polypeptin Sulfate

Original pH	Antibiotic activity, units per mg.						Hemolytic activity, times diluted					
	0 day	2 days	5 days	10 days	16 days	29 days	0 day	2 days	5 days	10 days	16 days	29 days
2.5	80	160	160	160	160	160	8000	8000	8000	8000	8000	8000
5.5	160	160	160	80	320	160	8000	8000	8000	8000	8000	8000
7.1	160	160	160	160	320	160	8000	8000	8000	8000	8000	8000
8.9	160	160	160	80	40	10	8000	8000	4000	2000	1000	<500
10.4	160	160	80	40	10	<10	8000	4000	2000	<500	<500	<500
11.5	160	160	20	10	<10	<10	8000	2000	<500	<500	<000	<500

peptin was slowly inactivated in the slightly alkaline solutions and rather rapidly inactivated in the more alkaline solutions. When inactivation occurred, both antibiotic and hemolytic activities were destroyed simultaneously. Loss of antibiotic and hemolytic activities in the more alkaline solutions was accompanied by disappearance of the turbidity and a decrease in the pH of the solution. The greatest loss in the activities recorded in Table IV accounts for approximately 90 per cent of the total. The inactivation of polypeptin may not have been produced by the alkali alone since the solutions were not sterile.

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SUMMARY

Polypeptin is a basic polypeptide which is isoelectric at pH 11.0. The sulfate, recrystallized from absolute methyl alcohol, appears to be a mix-

ture with a single component approaching 90 per cent. Many forms of crystals have been obtained by recrystallizing the sulfate from different solvents. The ultraviolet absorption bands of polypeptin sulfate indicate that polypeptin contains an unknown chromophore group or groups.

From data obtained by electrometric titration of polypeptin sulfate a minimum molecular weight of 2100 to 2200 was obtained, which is twice the apparent molecular weight obtained by the cryoscopic method. The ratio between the values for the acid and alkaline titrations of polypeptin, as well as the data for the amino nitrogen and sulfate content, indicates that polypeptin sulfate contains five or six amino groups to one carboxyl group.

The antibiotic and hemolytic activities of polypeptin are destroyed simultaneously by heating to the melting point and by incubation in alkaline solution. In the dry state or in weakly acid solution polypeptin and its salts retain their activity for at least 2 months.

The solubility of polypeptin sulfate in water and in salt solutions increases when the temperature is lowered; the solubility in mixtures of water and organic liquids increases when the temperature is raised. The sulfate is only temporarily soluble in anhydrous methyl and ethyl alcohols and appears to be precipitated from water solution by chloroform or ether, perhaps by the promotion of crystallization. A much more water-soluble form of polypeptin can be prepared by precipitation in an amorphous form from concentrated sodium acetate solution.

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